UNIVERSITY OF SHEFFIELD, MEDICAL SCHOOL

Department of Infection, Immunity & Cardiovascular Disease

Prostaglandin E2 Regulates Neutrophil Survival Via EP2/PKA/NR4A2 Signalling in the Context of COPD

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

by

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DEDICATION

To my grandma and my uncle,
from whom I inherited my first typewriter

EPIGRAPH

A peels an apple, while B kneels to God,
C telephones to D, who has a hand
On E's knee, F coughs, G turns up the sod
For H's grave, I do not understand
But J is bringing one clay pigeon down
While K brings down a nightstick on L's head,
And M takes mustard, N drives into town,
O goes to bed with P, and Q drops dead,
R lies to S, but happens to be heard
By T, who tells U not to fire V
For having to give W the word
That X is now deceiving Y with Z,
Who happens just now to remember A
Peeling an apple somewhere far away.

(Howard Nemerov)

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ABBREVIATIONS

Abbrev.	Name	Abbrev.	Name
15-keto PGE2	13,14-dihydro-15-keto PGE2	cAMP	Cyclic 3'-5'-cyclic adenosine monophosphate
15-(NAD)	Hydroxyprostaglandin dehydrogenase	CBR	Carbonyl reductases (i.e. CBR1, CBR2, CBR3)
15-oxo-PGE2	15-keto PGE2	CCL2,	Chemokine (C-C motif) ligand 2
15-PGDH	15-hydroxyprostaglandin dehydrogenase	CD	Cluster of differentiation (i.e. CD62L, CD11b, CD8)
5' AMP	5' adenosine monophosphate	cDNA	Complementary DNA
8-AHA-cAMP	8- (6- Aminohexylamino)adenosine cAMP	CMP	Common myeloid progenitors
A1AT	Alpha1 antitrypsin	CNB	Cyclic nucleotide binding domain
A2A	Adenosine A2A receptor	CNG	cAMP nucleotide-gated channels.
AA	Arachidonic acid	CNS	Central nervous system
AC	adenylyl cyclase	COPD	Chronic obstructive pulmonary disease
AF1	Activation function 1	Cox2	Cyclooxygenase-2
AMPK	AMP-activated Protein Kinase	CREB	cAMP response element-binding protein
APC	anaphase promoting complex	CRP	C-reactive protein
APS	Ammonium persulphate	CSE	Cigarette smoke extract
ARDS	Acute respiratory distress symptom	Csf2r	Colony stimulating factor 2 receptor
ATP	Adenosine triphosphate	CXCL	chemokines of the subgroup containing a CXC-motif
Bad	Bcl-2 associated death promoter	CXCR	CXCR1, 4
BAL	Bronchoalveolar lavage	CycB1	Cyclin B1
BALB/c	Bagg Albino c	CypB	Cyclophilin B
Bcl-2	B-cell lymphoma 2	DAMPs	Danger-Associated Molecular Patterns
Bcl-xL	B-cell lymphoma-extra large	DAPI	4',6-diamidino-2-phenylindole
BPI	Bacterial permeability-increasing protein	dbcAMP	Dibutyryl adenosine 3′,5′-cyclic monophosphate
BSA	Bovine serum albumin	DFP	diisopropyl fluorophosphate (DFP)
BTA	Butyric acid	DISC	Death-inducing Signalling complex
C57BL/1	Black 10 strain	dmPGE2	dimethyl PGE2 (dmPGE2)

Abbrev.	Name	Abbrev.	Name
DMSO	Dimethyl sulfoxide	HBSS	Hank's Balanced Salt Soln
DNA	Deoxyribonucleic acid	HC	Healthy control
dNTP	Deoxyribonucleotide triphosphate	HeLa	Henriette Lacks
D-PBS	Dulbecco's phosphate-buffered saline	HI-FCS	Heat-Inactivated Fetal Calf Serum
DPX	Distrene-80 plasticizer xylene	Hox	Homeobox (i.e. Hoxb8)
dsRNA	Double-stranded ribonucleic acid	HRP	Horseradish peroxidase
EC	Endothelial cells	HSC	Haematopoietic stem cells
EC50	Half maximal effective concentratio	ICAM	Intercellular adhesion molecule1
EDTA	Ethylenediaminetetraacetic acid	IFN β/γ	Interferon β/γ
EP	EP2 EP4	IKK	IκB Kinase
Epac	Exchange protein activated by cAMP	IL	Interleukin
ER	Estrogen-regulated	IP3	inositol 1,4,5-trisphosphate
ERK	Extracellular signal-regulated kinase	IRAK	Interleukin-1 receptor-associated kinase
EtOH	Ethanol	JNK	c-Jun N-terminal kinase
FAM	fluorophore 6-carboxylfluorescein	KD	Knockdown
FEV	Forced expiratory volume	Ki	Inhibitory constant
FRET	Fluorescence Resonance Energy Transfer (FRET)	LCP	myeloid lineage committed progenitors
fmlp	N-formyl-methionine- leucine-phenylalanine	LPS	Lipopolysaccharide
FSC	Forward scatter	L-selectin	CD62L
FVC	Forced vital capacity	LTB4	Leukotriene B4
fMLP	N-Formylmethionine-leucyl-phenylalanine	Mac-1	Macrophage-1 antigen
GAPDH	Glyceraldehyde-3-phosphate	MAPK	Mitogen-activated Protein Kinase
G-CSF	Granulocyte colony stimulating factor	MCF-7	Michigan Cancer Foundation 7
GM-CSF	Granulocyte-macrophage colony stimulating factor	Mcl-1	Myeloid cell leukaemia sequence 1
GOLD	Global Initiative for Chronic Obstructive Lung Disease	mCMP	Murine ER-Hoxb8 CMP
GoTAQ	Polymerase	MeOH	Methanol
G protein	guanosine triphosphate (GTP)-binding proteins	MMLV-	Moloney murine leukemia virus
GR-1	Myeloid differentiation antigen	MMP	Matrix metalloproteinase
GTP	Guanosine triphosphate	mNØ	Murine mCMP-derived Neutrophils

Abbrev.	Name	Abbrev.	Name
mTOR	Mammalian Target of Rapamycin	PKC	Protein kinase C
N6-MB-cAMP	N6-monobutyryladenosine cAMP	PLC	Phospholipase C
NADPHo	Nicotinamide adenine dinucleotide phosphate-oxidase	PMN	Polymorphonuclear leukocytes
NE	Neutrophil elastase	PMSF	Phenylmethylsulfonyl fluoride
NET	Neutrophil Extracellular Trap	PPARγ	peroxisome proliferator-activated receptor γ
NETosis	NET forming	PPP	Platelet poor plasma
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells	PS	Phosphatidylserine
Nor1	Neuronal orphan receptor 1	qPCR	Quantitative (real-time) PCR
NR4A	Nuclear family 4 subgroup A (NR4A)	QVD (Q-VD-OPh)	A carboxy terminal phenoxy group conjugated to valine and aspartate
Nurr1	Nuclear receptor related 1	Rap-1/2	Ras-related protein 1/2
Nurr77	Nuclear receptor related 77	Ras	rat sarcoma viral oncogene homolog
NSAIDs	Non-steroid anti-inflammatory drugs	RBC	Red blood cells
P2Y11	Purinoreceptor 2Y11	RCF (rcf)	Relative Centrifugal Force
PAF	Platelet activating factor	RISC	RNA-induced silencingcomplex
PBMC	Peripheral blood mononuclear cells	RNA	Ribonucleic Acid
PCR	Polymerase chain reaction	RNAi	RNA interference
PDE	Phosphodiesterase	RNAseq	RNA sequencing
PE	Phycoerythrin	ROS	Reactive oxygen species
PGD2	Prostaglandin D2	RPM	Revolutions per minute
PGDH	15-hydroxyprostaglandin dehydrogenase	RPMI	Roswell Park Memorial Institute medium
PGE2	Prostaglandin E2	RT-PCR	Reverse transcription PCR
PGES	Prostaglandin E synthase	RXR	Retinoid X receptors
PGF2α	Prostaglandin F2α	SAGE assay	Serial analysis of gene expression
PGH2	Prostaglandin H2	SCF	Stem cell factor
PGI2	Prostaglandin I2	SEM	Standard error of the mean
PI	Protease inhibitor	s-Lex	sialomucin sialylated Lewis-carbohydrate
PI3K	Phosphoinositide 3-kinase	siCasp-3	siRNA for Caspase3
PKA	Protein kinase A	siMcl-1	siRNA for Myeloid Cell Leukemia1

Abbrev.	Name	Abbrev.	Name
siRNA	Small interfering ribonucleic acid	TGFβ	Transforming growth factor β
SMC	Smooth muscle cells	TH17	T helper 17 cell
SSC	Side scatter	TLR	Toll-like receptor
STS	Staurosporine	TNFα,	Tumour necrosis factor-alpha
TAE	Tris acetate buffer	TRI reagent	Guanidinium thiocyanate-phenol-chloroform extraction reagent
TAMRA	tetramethylrhodamine	TXA2	Thromboxane A2
TBS	Tris buffered saline	VEGF	vascular endothelial growth factor
TEMED	Tetramethylethylenediamine		

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ABSTRACT

Chronic Obstructive Pulmonary Disease (COPD) is a neutrophil-associated pulmonary condition that is accompanied by chronic inflammation. Neutrophil lifespan is typically controlled by constitutive apoptosis, but their prolonged persistence at inflammatory sites is implicated in COPD pathology. However, current COPD therapies are inadequate at targeting neutrophilia.

Prostaglandin E2 (PGE2) is a potent pro-survival mediator in neutrophils that is increased in COPD. Therefore, PGE2 may contribute to neutrophilic accumulation in COPD lungs and the resolution of inflammation may be achieved by decreasing neutrophil lifespan. Specifically, it was hypothesised that PGE2 modulates protein kinase A (PKA)-dependent neutrophil lifespan through a specific prostaglandin (EP) receptor subtype, and that the nuclear receptors NR4A2 or NR4A3 are downstream effectors of PKA-mediated neutrophil survival.

Human polymorphonuclear neutrophils (PMN) were isolated from the blood of healthy volunteers and COPD patients. Cellular apoptosis was assessed by light microscopy and phosphatidyl serine exposure. Gene expression of PGE2 and NR4A receptors was measured by RT-PCR and qPCR. Additionally, a novel approach uses murine neutrophils (mNØ) derived from cultures of bone-marrow progenitors, conditionally immortalised by expression of a chimeric Hoxb8 protein. RNAi strategies to knock down *NR4A2* and *NR4A3* in mNØ were undertaken.

PGE2 and a pharmacological EP2 receptor agonist induced PKA-dependent PMN survival and *NR4A2* expression by qPCR. Likewise, PGE2-induced PKA survival was blocked by EP2 receptor antagonism. Interestingly, the inflammatory mediator LPS increased EP2 and EP4 expression by qPCR in healthy control and COPD PMN. Consistently, LPS induced early PKA-dependent survival and enhanced late PGE2 survival. Additionally, RNAi-mediated knockdown of *NR4A2* in mNØ decreased cell numbers during mNØ differentiation.

In conclusion, EP2 is the dominant receptor subtype in PGE2/PKA survival in PMN. In the context of COPD, LPS further enhances PGE2 survival through increased prostaglandin receptor expression. Moreover, this study links the nuclear receptor NR4A2 to neutrophil survival induced by the PGE2/PKA signalling axis, potentially providing a novel, specific molecular target for neutrophilia in COPD.

CHAPTER 1. CHRONIC OBSTRUCTIVE PULMONARY DISEASE: IMPACT OF NEUTROPHILS ON DISEASE PATHOLOGY.

Progressive lung-tissue destruction and remodelling in chronic inflammation are common characteristics of various otherwise distinct inflammatory lung diseases, such as chronic obstructive pulmonary syndrome (COPD), acute respiratory distress syndrome (ARDS), chronic asthma and cystic fibrosis. These conditions are estimated to cause 4.1 million deaths per year and will be the third most common cause of death in non-communicable diseases worldwide by 2030 (Abegunde *et al.*, 2007; WHO, 2007). Additionally, an estimated 210 & 300 million people suffer from COPD and asthma respectively (Masoli *et al.*, 2004, WHO, 2007). Tobacco abuse is a major risk factor for COPD and sensitisation in asthmatic disease development (Alwan *et al.*, 2010), but also exposure to second-hand smoke and air pollution can induce the disease (Abramson *et al.*, 2015; Assad *et al.*, 2015). Tobacco causes ~ 42 % of chronic respiratory diseases and tobacco-related deaths are estimated to increase to 10 % of total worldwide deaths by 2020 (Horton, 2007; Alwan, 2011). However, the susceptibility to the development of the disease varies amongst smokers and a substantial portion of patients are non-smokers (20 %; Mannino *et al.*, 2002; Lundback *et al.*, 2003). The only known genetic factor in the predisposition for the development of COPD is the mutation of the gene coding for the protease inhibitor α-1 antitrypsin. α-1 antitrypsin deficiency results in the development of COPD characteristics, mediated by the inefficiency to cleave neutrophil elastase (NE; de Serres, Blanco, 2012). Overall, inflammatory lung diseases, such as COPD, present an immense economic burden and are of major clinical importance, for smokers and non-smokers alike.

The severity and progression of the disease is categorized according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages (Rabe *et al.*, 2007). Spirometric assessment of lung function forms the basis of the classification of patient into 4 stages from a mild disease with limited sputum production and a chronic cough to a very severe clinical manifestation with secondary effects such as severely disabling exercise incapacity and cardiovascular complications (King, 2015). Airflow limitation is measured as a ratio of forced expiratory volume (FEV) and forced vital capacity (FVC). The progressive airflow limitation and shortness of breath (decrease in FEV) in the different stages may be interrupted by a sudden drop in FEV, which is classified as a disease exacerbation that may be life-threatening in stage IV disease patients (Rabe *et al.*, 2007). The processes leading to the decreased FEV values are well characterised: a destructive inflammatory process that ultimately results in peripheral airway narrowing and changes in the efficiency of gas exchange in emphysema and hypoxemia.

The COPD-characteristic inflammatory response (Pauwels *et al.*, 2001; MacNee, 2006) in the airways, pulmonary tissue and vasculature activated in response to the toxic xenobiotics and

free radicals contained in cigarette smoke (Yoshida, Tuder, 2007) is markedly different to the patterns of inflammation in other chronic inflammatory lung diseases, such as asthma (MacNee, 2006; Barnes, 2004). It is believed that cigarette smoke exposure of the epithelium and resident macrophages induces the production of chemoattractants (i.e. CXCL8 and LTB_4), pro-inflammatory cytokines and chemokines (Barnes, 2004). In the course of the immune response, a large number of effector cells, such as $CD8^+$ T-lymphocytes, neutrophils and monocytes are recruited from the circulation to the airways (Retamales *et al.*, 2001), which contribute to the secretion of chemokines, cytokines and proteinases (MacNee, 2006). Recruited and activated neutrophils induce the secretion of NE and metalloproteinases, contributing to the excessive mucus secretion, ultimately leading to the progressive obstruction of the airways, chronic bronchitis and airtrapping in the alveoli (**Fig. 1**). In acute exacerbations, the lung function deteriorates quickly and it has been reported that 14% of patients released from hospital after exacerbations die within a 3 month period (Roberts *et al.*, 2005). Exacerbations can be induced by bacterial, or viral respiratory infections, but also by environmental stimuli, such as air pollution and allergens. The number of neutrophils in the lung is even further increased during exacerbations (MacNee, 2006; **Table 1**), although the clearance of pathogens is impaired, leading to persistent or recurrent bacterial and viral infections and disease exacerbations. This may be caused by hypoxic stress, which can impair neutrophil apoptosis (Walmsley *et al.*, 2005), bacterial killing and has the potential to induce tissue damage due to increased degranulation (Hoenderdos *et al.*, 2013). The chronic inflammation in the small airways and the parenchyma ultimately results in the destruction of lung tissue and the development of emphysema, as well as fibrosis.

1.1. The Pathophysiology of COPD.

1.1.1. Implication of Neutrophils in the Pathophysiology of COPD.

The key inflammatory cells in COPD are thought to be neutrophils, which represent over 70 % of the cells found in the sputum of COPD patients (Peleman *et al.*, 1999). Neutrophil numbers in the lung increase with disease progression (Vestbo *et al.*, 2013; **Table 1**), and also during acute exacerbations (Qiu *et al.*, 2003). The increases were correlated with disease severity according to GOLD stages (Sparrow *et al.*, 1984; Keatings *et al.*, 1996; Stanescu *et al.*, 1996; Di Stefano *et al.*, 1998; Moermans *et al.*, 2011) and as such, neutrophil presence in the sputum was previously proposed to be included as a clinical marker of disease severity (Singh *et al.*, 2010).

Also increased are macrophages, which are often found in bronchoalveolar lavage (BAL) fluid or sputum and may represent over 20 % of cells found in COPD patient sputum (Peleman *et al.*, 1999), due to their increased presence in the airway lumen. In contrast, the

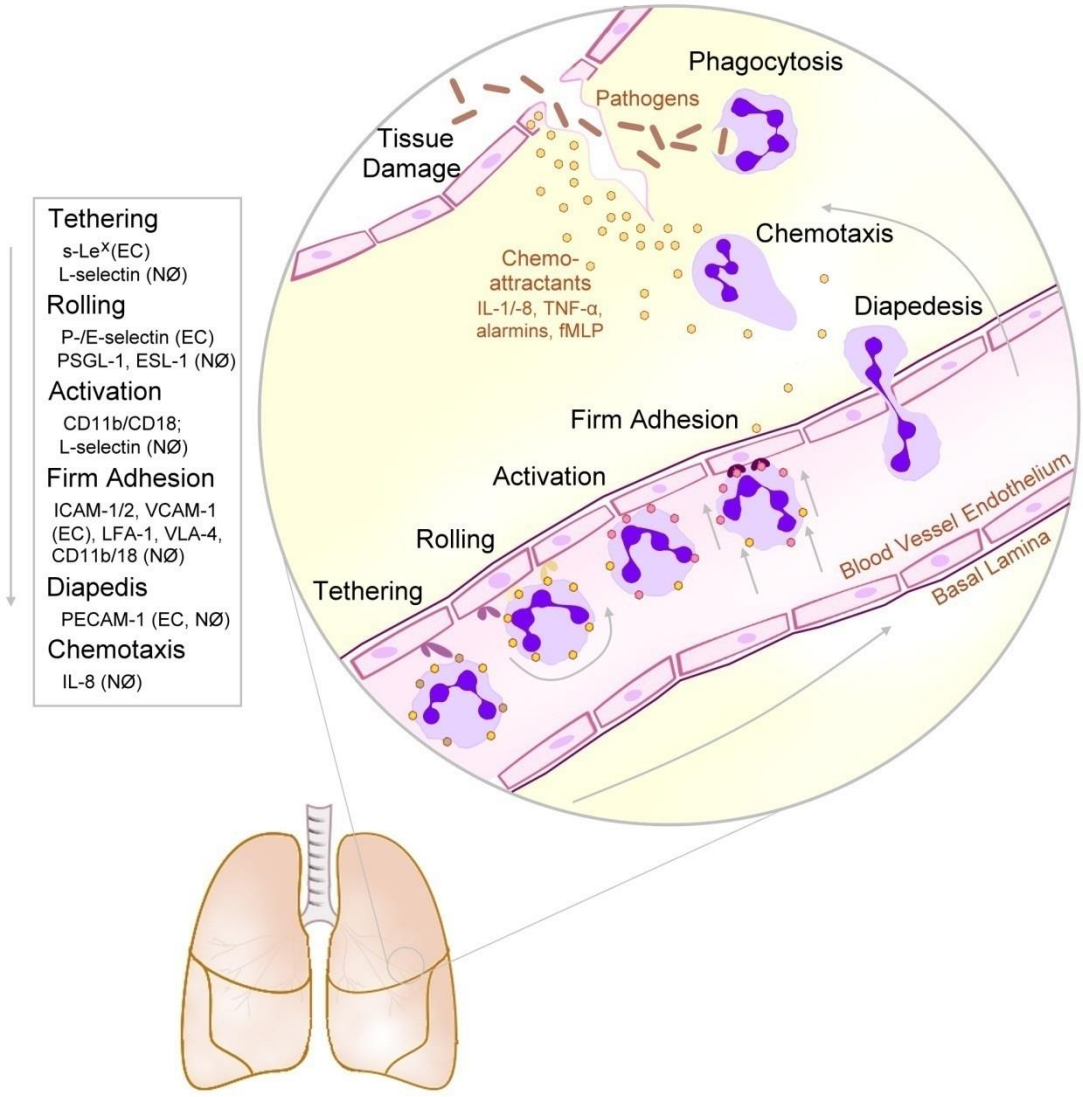


Figure 1: ***Neutrophil Recruitment Cascade.*** The recruitment of neutrophils is a multi-step process, following tissue damage or infection. After the release of neutrophils into the blood stream, they will be tethered by endothelial cells. Then, they roll along the endothelium and become activated close to sites of infection. Eventually, neutrophils will firmly adhere and then traverse the endothelial barrier and basal lamina in diapedesis and extravasation. Ultimately, they will chemotactically migrate towards the source of the chemokine gradient, released at the site of infection. During this process, neutrophils and endothelial cells will display different expression profiles, of which the most important are summarized in the box at the left.

Table 1: Evidence for a Role of Neutrophils in the Pathogenesis of COPD. The involvement of neutrophils in COPD pathology (overview of selected references only).

Cell types	Steady-state	Exacerbations	Contra
Increased Neutrophil Numbers	• Peleman *et al.*, 1999# • Baraldo *et al.*, 2004 (airway SMC) • Rutgers *et al.* 2000# • Lacoste *et al.*, 1993* • Keatings *et al.*, 1996# • DiStefano *et al.*, 1998†‡ (late-stage COPD)	• Qiu *et al.*, 2003† • Drost *et al.*, 2005* • Riise *et al.*, 1995*	• Qiu *et al.*, 2003† (stable/exacerbated) • Lacoste *et al.*, 1993‡ • Finkelstein *et al.*, 1995 (lung parenchyma) • Saetta *et al.*, 1993‡ • O'Shaughnessy *et al.*, 1997†
Increased Neutrophil Elastase	• Yoshioka *et al.*, 1999 • Panzner *et al.*, 2003	• Qiu *et al.*, 2003†	• Qiu *et al.*, 2003† (steady state)
NE Induces Mucus Hypersecretion or disease severity	• Nadel, 1991 • Hill *et al.*, 1999		
Increased Chemoattractants (and inflammatory markers)	• Keatings *et al.*, 1996# • Yamamoto *et al.*, 1997 • Traves *et al.*, 2002	• Qiu *et al.*, 2003† • Biernacki *et al.*, 2003 • Aaron *et al.*, 2001	• Qiu *et al.*, 2003† (steady state)
Correlation of Neutrophil Presence with COPD severity/ airway dysfunction	• O'Donnell *et al.*, 2004 • Baraldo *et al.*, 2004 • Peleman *et al.*, 1999 • DiStefano *et al.*, 1998	• O'Donnell *et al.*, 2004 • Baraldo *et al.*, 2004 • Drost *et al.*, 2005*	
Neutrophils correlate with emphysema	• Betsuyaka *et al.*, 1999* • Yoshioka *et al.*, 1995*		• O'Donnell *et al.*, 2004 • Finkelstein *et al.*, 1995
Delayed neutrophil apoptosis correlated with COPD severity	• Zhang *et al.*, 2012	• Zhang *et al.*, 2012	

BAL (*) - Broncho-alveolar lavage; SMC - smooth muscle cells; sputum (#); subepithelium bronchi biopsy (†); bronchial mucosa (‡)

increased presence of $CD8^+$ T-lymphocytes (Barnes, 2003) is not evidenced in BAL or sputum, as they commonly reside on the alveolar walls in the lung parenchyma.

1.1.1.1. Neutrophilia in COPD.

Neutrophils play a pivotal role in the pathogenesis of COPD. Neutrophil presence is increased by over 30 % in the induced sputum of COPD patients (Perng *et al.*, 2004), and neutrophilia in both stable-state COPD and exacerbations are a well documented occurrence (see **Table 1**). Despite the compelling evidence for the detrimental effects of neutrophils in chronic inflammation of COPD *in vivo* (O'Donnell *et al.*, 2004; Baraldo *et al.*, 2004), neutrophilia remains a particularly neglected characteristic of COPD pathology. Firstly, neutrophil secretions, such as NE are increased (Damiano *et al.*, 1986; Gottlieb *et al.*, 1996) and have been linked to induction of mucus hypersecretion (Nadel, 1991) and emphysema (Galdston *et al.*, 1977; Yoshioka *et al.*, 1995). Neutrophil presence (Perng *et al.*, 2004) and activation (Caramori *et al.*, 2005) was correlated with deteriorating lung function and the severity of the symptoms, respectively.

1.1.1.2. Neutrophilic Inflammation

Neutrophils are thought to perpetuate chronic inflammation in COPD, through the secretion of inflammatory cytokines or the co-incidental release of cytotoxic substances, such as of NE or reactive oxygen species (ROS), leading to tissue damage. (MacNee *et al.*, 1989; Dubravec *et al.*, 1990; Tetley, 1993; Baines *et al.*, 2011).

Neutrophils became invariably linked to the inflammatory phenotype of COPD and its pathology, as the genetic disease α1-antitrypsin deficiency was shown to cause emphysema in a COPD patient cohort (Laurell *et al.*, 1963). α1-antitrypsin is the major protease inhibitor of NE and alterations in the balance of proteases and antiproteases in the lung are thought to facilitate neutrophil-induced tissue damage (Tetley, 1993). In this context, neutrophilic NE content and mucus hypersecretion (Nadel, 1991), as well as the severity of emphysema in COPD have long been linked (Galdston *et al.*, 1977; Yoshioka *et al.*, 1995). NE and other proteases, such as cathepsin G, matrix metalloproteinases (MMP) and proteinase 3 can thereby stimulate the general mucosal secretion by mucus glands and goblet cells in the pulmonary epithelium by direct and indirect mechanisms (Sommerhoff *et al.*, 1990; Tetley *et al.*, 1993; Witko-Sarsat *et al.*, 1999; O'Donnell *et al.*, 2006), resulting in progressive airway obstruction and lung damage in chronic bronchitis.

The increased serine protease content in inflammatory neutrophils (Burnett *et al.*, 1987; Nadel, 1991) may thus contribute to the increased proteolytic capabilities of neutrophils from emphysema patients (Burnett *et al.*, 1987; Betsuyaku *et al.*, 1999). A prerequisite for the immune function of neutrophils is their functional activation.

Priming is required for the functional activation of neutrophils, and recruited neutrophils in the COPD lung are likely to be activated, as evidenced by the increased presence of neutrophil products in COPD BAL (Condliffe *et al.*, 1998; Noguera *et al.*, 2001; Keatings, Barnes, 1997; Peleman *et al.*, 1999). Moreover, COPD neutrophils show increased responsiveness to inflammatory mediators, such as LPS and in the respiratory burst (Brown *et al.*, 2009; Langereis *et al.*, 2011; Blidberg *et al.*, 2012; Richards *et al.*, 1989).

1.1.1.3. Mechanisms in the Modulation of Pulmonary Neutrophilia.

As elaborated above, neutrophil presence in COPD is increased and their presence contributes to the development of all major disease characteristics and is has been described to perpetuate chronic inflammation. Neutrophil proteinase secretions induce mucus hypersecretion, bronchitis and fibrosis. Increased neutrophil numbers may be caused by four distinct variables that may contribute to the neutrophilic phenotype of COPD. Firstly, accelerated neutrophil maturation and increased release from the bone marrow; secondly, increased recruitment and neutrophil infiltration into the pulmonary vasculature, tissues and airways; thirdly, the prolongation of their lifespan in the inflammatory environment and lastly, a failure of apoptotic neutrophil clearance.

Left-shifts, i.e. a rapid increase in the bone-marrow release of immature neutrophils into the bloodstream, are a highly specific indicator of infections (Seebach *et al.*, 1997), being attributed to the increased need for neutrophils in an inflammatory setting. The level of neutrophil maturity relates to the expression of cell surface receptors, such as CXCR4, which can cooperate with inflammatory chemokines to orchestrate neutrophil release in inflammation (Suratt *et al.*, 2004). Although the expression of cell surface receptors in COPD peripheral blood neutrophils, such as TLR2, cell adhesion molecules and chemokine receptors, has repeatedly been found to be altered (von Scheele *et al.*, 2011; Kennedy, Kilpatrick, 2013), no potential alterations in neutrophil maturity have been investigated, or linked to this occurrence. However, it has been shown that smoking may induce the release of neutrophils from the bone marrow (Terashima *et al.*, 1997), possibly mediated by the release of inflammatory mediators by alveolar macrophages upon phagocytosis of carbon particles.

Evidence for increased neutrophil infiltration of the COPD lung tissue was indirectly provided by the massive presence of chemoattractants (further discussed in chapter 1.1.2) and by the increased expression of the adhesion molecule E-selectin on pulmonary endothelial cells (Di Stefano *et al.*, 1994). However, neutrophil presence was predominantly found in the BAL or sputum of COPD smokers (Rytilä *et al.*, 2006; Makris *et al.*, 2009; Pace *et al.*, 2011), whereas their increased presence in pulmonary tissue could not consistently be demonstrated by immunohistochemical methods (Finkelstein *et al.*, 1995; Retamales *et al.*, 2001). This may suggest that the main determinant for their accumulation in the lung appears to be subsequent to the recruitment step.

Neutrophil survival in the airways may become increased due to the presence of inflammatory survival factors, such as LPS and PGE2 (further discussed in chapters 1.1.2 and 1.3), which enables their accumulation in the lung. Moreover, it was proposed that the functionality of phagocytic macrophages is reduced due to noxious substances in cigarette smoke (Terashima *et al.*, 1997; Lundborg *et al.*, 2001; Lundborg *et al.*, 2006), resulting in the defective clearance of neutrophils from the airways. Since compelling evidence for both scenarios exists, it is likely that both cooperate in the occurrence of neutrophilia in COPD.

1.1.2. Inflammatory Mediators in the Pathophysiology of COPD

Chronic Inflammation in COPD increases the pulmonary levels of various cytokines, chemokines and prostaglandins that have known immunomodulatory properties. While being a typical inflammatory response, the pulmonary microenvironment is unique in regards to the influences of oxygen, exposure to environmental pollutants and bacterial products, such as lipopolysaccharide (LPS). Airway inflammation increases the susceptibility to disease exacerbations and therefore, the events and modulators in chronic COPD inflammation are of major importance. This chapter will explore the influence of inflammatory mediators on COPD pathology and their role in interactions with other cells in the lung parenchyma.

1.1.2.1. Chemoattractants & Cell Activators

One of the first stages of injury following vasodilation is the influx of immune cells into the tissue via the release of chemotactic factors at the site of inflammation. Major migratory factors released in the context of inflammation are Leukotriene B4 (LTB4), CXC motif chemokine 8 (CXCL8; formerly IL8) and the CXC3 subfamily of chemokines (Tecchio *et al.*, 2014). Both LTB4 and CXCL8 are increased in COPD (Traves *et al.*, 2002; Tanino *et al.*, 2002) and cytokine levels correlate with neutrophil numbers (Profita *et al.*, 2005; Perng *et al.*, 2004). Further studies have confirmed the chemotactic effects of these mediators on neutrophils (Corhay *et al.*, 2009; Beeh *et al.*, 2003). The major sources of LTB4 are thought to be activated macrophages and neutrophils (Shamsuddin *et al.*, 1997; Bazzoni *et al.*, 1991). CXCL8 is thought to be released in the context of COPD by a variety of activated cell types, such as epithelial cells, macrophages, neutrophils and smooth muscle cells (de Boer *et al.*, 2000; Zhang *et al.*, 1995; Altstaedt *et al.*, 1996; Oltmanns *et al.*, 2005). In particular, neutrophilic cytokine release may in this context contribute to the self-perpetuating loop of inflammation in COPD.

The CXCL cytokines CXCL9, CXCL10 and CXCL11 are major $CD8^+$ T lymphocyte attractants that are elevated in COPD (Clark-Lewis *et al.*, 2003; Panzner *et al.*, 2003; Saetta *et al.*, 2002; Porter *et al.*, 2008). Moreover, the corresponding ligand receptor CXCR3 is overexpressed on T lymphocytes in COPD smokers (Saetta *et al.*, 2002). However, while CXCL9 and CXCL10 may contribute to the accumulation of T lymphocytes in the lung (Barnes, 2004), CXCL11 has

also been found to be involved in the resolution of inflammation as CXCL11 levels are decreased in the sputum of patients with fibrosis and emphysema (Zhao *et al.*, 2012) and also by establishing a cytokine gradient in egression (Porter *et al.*, 2008). Due to their potentially dual roles in cell recruitment and resolution of inflammation, the classification of cytokines as pro- or anti-inflammatory stimuli is complicated. Moreover, chemoattractants can possess additional functions, as cell activators and pro-survival mediators, as for platelet activating factor (PAF), which attracts and activates neutrophils, but is not expressed in COPD (Barnes, 2004).

1.1.2.2. Pro-Survival Mediators

As a second step in the inflammatory response, the lifespan of immune cells in the lung can be extended by the presence of inflammatory mediators with potent pro-survival properties in the respiratory tract. In this context, macrophage and even more so neutrophil survival are sensitive to pro-survival factors. Here, the influence of LPS and selected cytokines will be explored.

LPS

LPS is a membrane component of gram negative bacteria. *In vivo*, LPS causes a COPD-like phenotype, as LPS-challenge exacerbates elastase-induced emphysema in mice and induces the secretion of the COPD biomarkers C-reactive protein (CRP), NE and α1-antitrypsin (Kobayashi *et al.*, 2013; Korsgren *et al.*, 2012). LPS acts via TLR4 receptors, which are expressed on neutrophils, and are upregulated on T cells in COPD (Sabroe *et al.*, 2002a; Sabroe *et al.*, 2003; Freeman *et al.*, 2013). In neutrophils, LPS is a potent activator and pro-survival molecule and causes MAPK activation, CD62L shedding, CD11b expression, respiratory burst and cytokine generation (Sabroe *et al.*, 2002a; Sabroe *et al.*, 2003; Dick *et al.*, 2009). In COPD neutrophils, the LPS-induced secretion of CXCL8 and MMP, as well as mRNA expression of TLR2 and TLR4 is enhanced, suggesting that COPD neutrophils might be more susceptible to LPS challenge or bacterial infections (Baines *et al.*, 2011). In macrophages, LPS induces the secretion of a variety of inflammatory mediators, such as IL-6, IL-10 and IFNβ (Strassmann *et al.*, 1994; Yamane *et al.*, 2000; Xu *et al.*, 2008; Finney-Hayward *et al.*, 2009; Rossol *et al.*, 2011), by which it may perpetuate chronic inflammation in the condition. LPS has also been shown to induce the secretion of the prostaglandin PGE2 by the engagement of cyclooxygenase-2 (Cox2) in macrophages and endothelial cells (Ulcar *et al.*, 2004; Amann *et al.*, 2001; Ikegami *et al.*, 2001; Hofer *et al.*, 2004; Yamagata *et al.*, 2001), potentially through the activation of nuclear factor NF–κB (Newton *et al.*, 1997; Andreakos *et al.*, 2004). This is thought to contribute to increased levels of PGE2 in inflammatory settings and the self-perpetuating characteristics of COPD.

Growth Factors

Although interleukins and related cytokines may have some protective properties on cell survival, a central role for growth factors, such as granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) was found (Coxon *et al.*, 1999; Jun *et al.*, 2015). In particular, GM-CSF was able to delay neutrophil apoptosis, induced by the cytokine-mediated activation of endothelial cells (Coxon *et al.*, 1999) with NFκB being a major inducer of GM-CSF synthesis (O'Donnel *et al.*, 2006). Macrophages are also able to secrete GM-CSF (Culpitt *et al.*, 2003). In COPD, the levels of GM-CSF are increased (Balbi *et al.*, 1997) and may thus potentially have a role in COPD inflammation. Moreover, the vascular endothelial growth factor (VEGF) had a role in alveolar cell survival, whereas its levels in COPD were not found to be increased (Kasahara *et al.*, 2000).

1.1.2.3. Oxidative Stress

The balance of oxidants and antioxidants in the lung has also been found to be disturbed in COPD and that this is an important characteristic of the disease (Stanojkovic *et al.*, 2011; MacNee, 2001). Major oxidants are the reactive oxygen species (ROS), which are contained in cigarette smoke and can also be released by neutrophils and macrophages in response to inflammatory stimuli, such as LPS (MacNee, 2001; Barnes, 2004; Bae *et al.*, 2009). ROS alters the protease balance by the activation of proteases, such as NE and inhibition of the antiprotease A1AT. Coincidentally, ROS is linked to the secretion of the cytokines CXCL8 and TNFα (Zinovkin *et al.*, 2014), which are potent pro-inflammatory agents in COPD. As ROS prevents macrophage phagocytosis, it was also previously linked to the efficient clearance of bacteria from the COPD lung (Thimmulappa *et al.*, 2012; Yoshida *et al.*, 2007). Moreover, it has been shown that ROS has the potential to induce direct tissue damage in the COPD lung (Neofytou *et al.*, 2012).

1.2. Investigating Neutrophil Biology in the Context of COPD.

The importance of neutrophils in the immune response is clearly demonstrated by the effect of their dysregulation. Low neutrophil levels in neutropenia are correlated with increased persistent infection risk and high mortality, while neutrophilia is often observed in infection, where increased amounts of neutrophils are required to match the infection size. In this sense, tight regulation of neutrophil levels is necessary and this is reflected in their spontaneous death after a short lifespan. Increased amounts of bacterial and viral lung infections are observed during exacerbations in the advanced stages of COPD. Correlations between elevated neutrophils, bacterial or viral exacerbations and deteriorating lung function might support either physiological or pathogenic roles of neutrophils in this setting (Toews, 2005). These might account for neutrophil recruitment, but not necessarily for their persistence in the chronic

inflammatory response in the lung. Upon infection, neutrophil lifespan is prolonged and is often further modulated in chronic disease (Ertel *et al.*, 1998; Jimenez *et al.*, 1997).

In the chronic inflammatory settings of chronic asthma, COPD and cystic fibrosis, increased chemokine expression and chemokine release through tissue damage is present (Drost and MacNee, 2002), acting as potential neutrophil chemoattractants. The main models of self-amplifying neutrophil recruitment that fails to clear a persistent bacterial infection are twofold (Hartl *et al*, 2007). In cystic fibrosis, proteolytic CXCR1 cleavage inactivates neutrophils and triggers CXCL8 expression in epithelial cells (Hartl *et al*, 2007, Sabroe and Whyte, 2007). Mucus hypersecretion and resulting elastase-mediated lung injury may predispose for infections and thus increase neutrophil recruitment. Although progressive tissue damage in COPD is often attributed to the neutrophilic release of cytotoxic contents in the immune response (Coxon *et al.*, 1999; Savill *et al.*, 2000; Parker *et al.*, 2005), exaggerated neutrophil presence in COPD was also found in absence of bacterial colonisation of the lung (Makam *et al*, 2009).

1.2.1. Neutrophil Maturation, Recruitment and Immune Roles in the Lung.

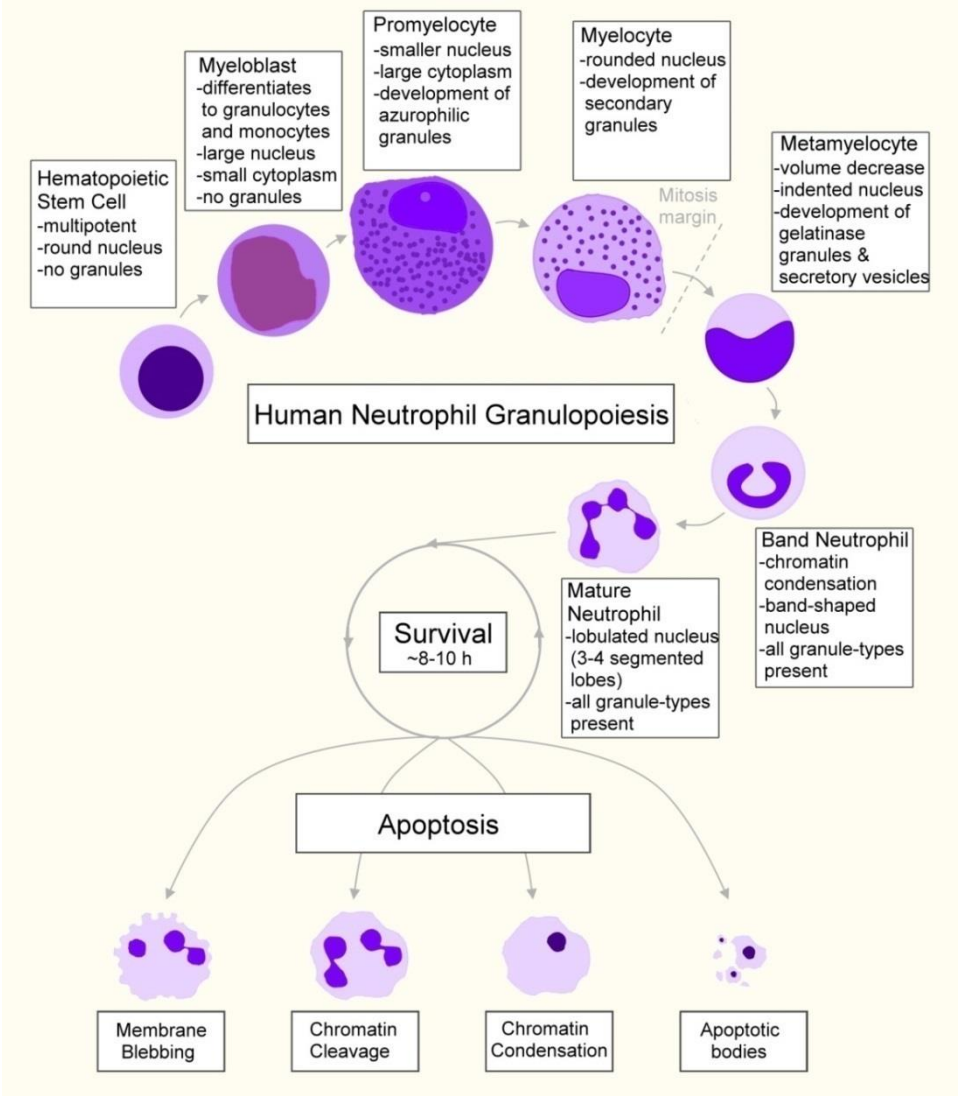
In the course of the innate immune response, neutrophils are the first recruited immune cells at inflammatory sites. Their recruitment proceeds through a series of consecutive events, beginning with the margination, tethering, rolling, activation and firm adhesion of neutrophils on the blood vessel endothelium, followed by diapedesis, crossing of basal lamina and subsequent chemotactic migration towards the source of an inflammatory chemokine gradient. Margination occurs independently of infection, while rolling is more commonly associated with infection. Following their recruitment, neutrophils phagocytose pathogens and release extracellular traps (NETosis) to capture and destruct pathogens at the site of infection.

1.2.1.1. Haematopoiesis

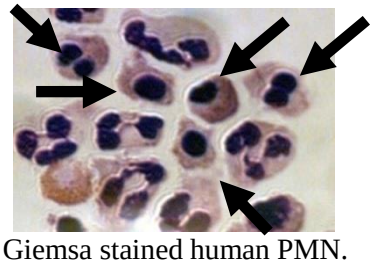
Neutrophils are derived from multipotent myeloid, hematopoietic stem cells of the bone marrow, which differentiate in a multi-stage process termed granulopoiesis, upon stimulation with the survival sustaining cytokine granulocyte colony-stimulating factor (G-CSF; Basu *et al*, 2002). Granulopoiesis is a continuous process taking up to 14 days, where cells remain mitotic until the myelocyte stage at approximately day 7 (Bainton *et al*, 1971) after which time the characteristic nuclear changes occur.

Neutrophils mature in six stages, namely myeloblasts, promyelocytes, myelocytes, metamyelocytes, band neutrophils and finally mature neutrophils (**Fig. 2**). The presence of four granule types in neutrophils is essential for their immune function. Azurophilic granules develop during promyelocyte stages, followed by secondary granules in myelocytes (Bainton *et al*, 1971). In metamyelocytes, gelatinase granules and secretory vesicles develop (Jog *et al*, 2007). During phagocytosis, azurophilic granules fuse with phagocytic vesicles, where granule

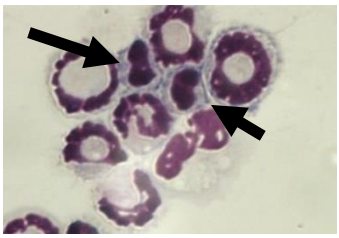
A



B



Giemsa stained human PMN.



Giemsa stained murine mNØ.

Figure 2: ***Neutrophil Lifetime and Development.*** Human neutrophil granulopoiesis takes place in the bone marrow. Initially, common myeloid progenitors develop into myeloblasts, promyelocytes and myelocytes during the first developmental week, where they can still divide (Panel **A**). During the second week, post-mitotic development to metamyelocytes, band and mature neutrophils takes place. Characteristic features of these stages are summarized in boxes. The boundary between pre- and post-mitotic development is highlighted as mitosis margin and a discontinued line. After their survival in the circulation (8-10 hours), neutrophils die through apoptosis. Common characteristics are membrane blebbing, chromatin cleavage and condensation and formation of apoptotic bodies (Panels **A**, **B**).

contents, consisting of defensins, lysozyme, azurocidin, bacterial permeability-increasing protein (BPI) and elastase, mediate the destruction of diverse kinds of pathogens. Secondary granules are secreted outside of the cell in exocytosis and notable contents are vitamin B12-binding protein, lysozyme and collagenase. Gelatinase granules, as well as secretory vesicles secret their content to the neutrophilic cell-surface. While the former contain the movement-facilitating gelatinase, acetyltransferase and lysozyme, the latters' most notable contents are fMLP-receptors, which are important in pathogen-derived signalling. Thereafter, nuclear chromatin condensation forms a band-like structure. Nuclei of mature human neutrophils form lobulated nuclei with 3-4 segmented lobes, connected through chromatin filaments.

1.2.1.2. Neutrophil Recruitment

Innate immune responses are initiated by tissue injury or infection. Thus damaged, epithelial cells activate local inflammatory cells, such as macrophages through chemokine release (as described in chapter 1.1.1.). This might increase vasodilation and enhance vascular permeabilisation, leading to the inflammatory side effects of swelling and pain. Further chemokine release (TNFα, IL-1/IL-6) of local macrophages activates neighbouring blood vessel EC, enabling capturing of blood neutrophils and facilitated recruitment through the permeabilised membranes towards the chemokine gradient (Feldmann *et al*, 1996, Janeway *et al*, 2001).

The neutrophil recruitment cascade initiates with the margination of neutrophils along the blood vessel endothelium, as the forces of the blood stream will initially push them towards the vessel wall, where they will be tethered by endothelial cells (EC). Once captured neutrophils have formed an initial bond, they will migrate along the blood vessel wall in a rolling motion through consecutive release and readhesion to EC through strong, but transient ligand-receptor binding of the constitutive endothelial sialomucin sialylated Lewis-carbohydrate (s-Lex). This selectin ligand mainly binds to neutrophil L-selectin, which is expressed in higher levels, directly after release of neutrophils into the circulation (Matsuba *et al*, 1997). However, inhibition of L-selectin shedding does not only increase the amount of firm adhesion, but also of neutrophil extravasation *in vivo* (Hafezi-Moghadam *et al*, 2001), implicating L-selectin in neutrophilic transmigration. The specific role of L-selectin is still controversial, due to contradictory studies *in vitro* and *in vivo* (Allport *et al*, 1997, Hafezi-Moghadam *et al*, 2001).

The capturing of rolling neutrophils and their firm binding to the endothelium is enabled through the activation of EC through local inflammatory cytokines, i.e. TNFα, CXCL1, leading to upregulation of P-/E-selectin expression and further chemoattractant chemokines in EC. The presented chemokines are specific to the immune cells they are recruiting. Neutrophils are specifically recruited by specific G-coupled protein receptors through the endothelial chemokines of the subgroup containing a CXC-motif close to an ELR-motif (i.e. CXCL1-3;

CXCL5-8; Murdoch and Finn, 2000; Laing and Secombes, 2004). Moreover, additional binding of endothelial CD54 (ICAM-1) to the adhesion molecules CD11b/CD18 (Smith *et al*, 1989) results in the arrest of rolling neutrophils. Thereafter, neutrophils migrate through EC, or intercellular junctions, to enter the tissue, where they migrate chemotactically towards the source of the released CXCL8 gradient. The major routes of migration for neutrophils into the pulmonary spaces are thought to be through the alveolar walls and postcapillary venules in the tracheobronchial tree (Hogg, Walker, 1995; Pettersen, Adler, 2002), whereas potential alterations in the cellular mechanisms of neutrophil recruitment through the pulmonary vasculature are not well established and subject to intensive research. The rearrangement of the neutrophil actin cytoskeleton and the polarisation of the cell play crucial roles in leading edge formation, required for the detachment of the cell from its former integrin bonds, chemotaxis and subsequent phagocytosis of pathogens (Moissoglu and Schwarz, 2006, Servant *et al*, 2000).

Neutrophil activation coincides with their recruitment by EC following the rolling stage, which upregulates the integrins CD11b/CD18 and leads to L-selectin shedding. Activated neutrophils can potentiate neutrophil and monocyte recruitment through the release of the chemoattractants CXCL1, CXCL2, CXCL8, CCL2, CCL3 and CCL4, which have also pro-inflammatory properties (Scapini *et al*, 2000; Hurst *et al*, 2001). In turn, recruited monocytes can prolong neutrophil lifespan through the release of the survival factors G-CSF, GM-CSF and low amounts of TNFα (Silva, 2010), additionally enhancing neutrophil presence.

1.2.1.3. Pathogen Clearance

Phagocytosis is an important mechanism of neutrophil-mediated pathogen destruction, where neutrophils engulf pathogens and enclose them in phagocytic vesicles. The phagocytic response to bacterial pathogens is primarily engaged due to the binding of TLR2, TLR4 and TLR5 to lipoproteins, LPS and flagellin, respectively (Parker *et al.*, 2005). Azurophilic granules fuse with the phagocytic vacuole to form a phagolysosome. Additionally, activation of cells, e.g. via fMLP receptors, triggers a respiratory burst in response to pathogens. During this response, NADPH oxidase generates superoxide radicals, which are transformed to hydrogen peroxide and then ultimately to hydroxyl radicals under oxygen consumption. These reactive oxygen species (ROS) are then released into the phagocytic vesicles (Edwards, 1994). Interestingly, monocyte/macrophage and polymorphonuclear cell phagocytosis was impaired in COPD patients (Prieto *et al.*, 2001; Taylor *et al.*, 2010), mimicked by the exposure to first- and second-hand cigarette smoke (Martí-Lliteras *et al.*, 2009; Ni *et al.*, 2015), carbon particles (Lundborg *et al.*, 2001; Lundborg *et al.*, 2006) or the modelling of an inflammatory environment in COPD (McPhillips *et al.*, 2007). These factors may contribute to impaired macrophage efferocytosis of neutrophils, comparable to their reduced efferocytosis of eosinophils (Eltboli *et al.*, 2014).

Moreover, neutrophil survival determines their inflammatory function (Whyte *et al.*, 1993a; Watson *et al.*, 1996; DeLeo *et al.*, 2004).

A second mechanism of neutrophilic immune responses upon stimulation with bacterial endotoxin LPS or the cytokine CXCL8, is the release of chromatin fibre nets, which act as extracellular traps and barriers for pathogens (Brinkmann *et al*, 2004). Neutrophil Extracellular Traps (NETs) are constituted of released DNA, histones, serine proteases and elastase – all of which are implicated in neutrophil-mediated inflammation and tissue damage. This is a broadly targeted process, but risks the release of tissue damaging factors (Lee and Grinstein, 2004). In particular, NE was shown to affect tissues in COPD, which directly relates presence of NETs with tissue damage. In cystic fibrosis, NETs have also been implicated in mucus build-up, as the treatment of cystic fibrosis lungs with the NET-destroying enzyme DNAse leads to considerable improvement in mucus clearance (Fuchs *et al*, 1994).

1.2.2. Resolution of Inflammation: The Mechanisms of Neutrophil Lifespan Regulation.

Apoptotic, but not necrotic neutrophil death prevents the release of tissue damaging compounds that are required by neutrophils for pathogen clearance. Apoptosis can be engaged by external stimuli, such as ligand binding to the Fas receptor, or through pathogen-mediated stimuli through the TNF receptor. The intrinsic pathway can be activated by irradiation, however also passive effects, such as the withdrawal of growth factors results in the development of an apoptotic phenotype. Neutrophils are unique, as they furthermore undergo constitutive apoptosis that predetermines their lifespan. However, a loss of functionality is observed in the course of neutrophil apoptosis. In particular, neutrophils may lose their ability to phagocytose, chemotax, degranulate and participate in cytokine secretion (Whyte *et al.*, 1993a; Watson *et al.*, 1996; DeLeo *et al.*, 2004). Thus, upon fulfilment of their immune function, neutrophils are cleared from the infected site, as they may otherwise prolong inflammation (Savill *et al.*, 2000). Apoptotic neutrophils can be cleared from the site of inflammation by macrophage efferocytosis through the exposure of the cell surface epitope phosphatidylserine (PS; Savill *et al.*, 1989; Fadok *et al.*, 1992), or through their reverse migration to the spleen (Mathias *et al.*, 2006, Buckley *et al.*, 2006), contributing to the maintenance of adequate amounts of neutrophils in inflammatory tissues.

1.2.2.1. Regulation of Neutrophil Clearance

Apoptosis

Apoptosis was first described as an important phenomenon in the course of embryogenesis, where biochemical and morphological changes in the cell are actively engaged to remove cells, while necrosis is more often seen as a failure of cells to maintain homeostasis. Neutrophils were

long thought to only survive ~8-10 hours following their maturation and release from the bone marrow (Basu *et al*, 2002; Bicknell *et al.*, 1994). However, a more recent re-evaluation of neutrophil lifespan showed that their lifespan in circulation might be up to 10 times longer than previously reported (Pillay *et al*, 2010). The lifespan of peripheral blood neutrophils is limited through constitutive apoptosis and thereby significantly shorter than that of other immune cells. The recruitment of neutrophils to inflammatory sites functionally activates them by inflammatory mediators, such as G-CSF and GM-CSF (Saba *et al.*, 2002) to promote pathogen clearance. Thereby, their lifespan is prolonged to 1-4 days (Colotta *et al.*, 1992; Brach *et al.*, 1992), preventing the resolution of inflammation. Inflammatory tissue damage may thus be caused by a neutrophilic failure to engage apoptosis (Savill *et al.*, 2000), substantiated by delayed neutrophil apoptosis in various malignancies, such as inflammatory bowel disease (Dibbert *et al*, 1999, Brannigan *et al*, 2000). Therefore, upon clearance of pathogens from the site of infection, neutrophils have fulfilled their immune functions, are obsolete and need to be removed. This mainly occurs through spontaneous apoptosis, which facilitates their phagocytic removal through macrophages (Savill *et al*, 2002). In contrast to necrosis, apoptotic cell death can occur under normal physiological conditions, and requires the cell to actively participate in its own death by activation of cellular signalling chains and machinery. Additionally, a novel mechanism of neutrophil clearance has been proposed to be neutrophilic reverse migration.

Morphologically, apoptotic neutrophils are characterised by cleavage of inter-lobular chromatin bridges leading to nucleus and chromatin condensation, cytoplasmic vacuolisation, membrane blebbing and eventually, formation of apoptotic bodies, which contain fragments of the nucleus (**Fig. 2**). In contrast to necrosis, DNA degradation in apoptosis is a coordinated process, as the DNA is cleaved into a distinctive pattern by endonucleases. Prior to the morphological changes, which ultimately allow the efferocytosis of neutrophils, surface receptor changes and alterations in the biochemistry take place. Furthermore, exposure of phospholipids such as phosphatidylserine (PS) on the cell surface, allows the selective recognition of apoptotic neutrophils for their clearance by macrophages (Fadok *et al*, 1992, Savill *et al*, 1989). While necrotic cells swell and rupture, apoptotic neutrophils maintain their membrane integrity with decreasing cell volume and do not elicit an inflammatory response, as they become phagocytosed before the release of cytotoxic intracellular contents. However, in part, this is also due to the activation of TGFβ, which decreases the inflammatory response through downregulated cytokine production in apoptotic neutrophils and anti-inflammatory cytokine expression by macrophages following neutrophil efferocytosis (McDonald *et al*, 1999). No pro-inflammatory substances are released from the cell in apoptotic death, thereby circumventing potentially additional tissue damage to surrounding tissue by an inflammatory response.

Neutrophil apoptosis can be inhibited by multiple factors to allow bacterial clearance *in vivo* (Coxon *et al*, 1996, Dibbert *et al*, 1999, Hotta *et al*, 2001). Pro-inflammatory cytokines,

such as GM-CSF and G-CSF (see chapter 1.1.2.2.) prolong the survival of neutrophils at sites of inflammation. However, even the anti-apoptotic effect of the most important survival factor, granulocyte-macrophage colony stimulating factor (GM-CSF) is only limited and cannot entirely reverse the fate of neutrophils.

Apoptosis can be modulated through various molecular mechanisms, but is always accompanied by a downregulation of neutrophil function. This was shown by the inability of apoptotic neutrophils to initiate a respiratory burst upon stimulation and their degranulation (Savill, 1997a; Whyte *et al*, 1993a). Lost chemotactic abilities were also observed and attributed to inhibited polarisation through the actin cytoskeleton, as well as inhibition of granule exocytosis (Jog *et al*, 2007). Apoptotic neutrophils shed various adhesion molecules and surface receptors (Jones and Morgan, 1995, Whyte *et al*, 1993a). Moreover, it was previously reported that the inflammatory environment may enhance PS cleavage (Vandivier *et al.*, 2002; Huynh *et al.*, 2002) and therefore inhibit immune cell clearance from the lung (Fadok *et al.*, 2000).

Reverse Migration

Reverse migration is a common and well-described process in various immune cells, such as monocytes and lymphocytes (Bradfield *et al*, 2007, McGettrick *et al*, 2009, Randolph *et al*, 1998), all of which possess significantly longer lifespans than neutrophils, enabling them to be recirculated and potentially re-used in novel inflammatory responses. Despite their hypothesised potential for only a single respiratory burst, the underestimation of their lifespan challenges the general validity of apoptotic neutrophil clearance by macrophages (Pillay *et al*, 2010; Buckley *et al*, 2006). Neutrophil populations that are present in inflammatory conditions have been found to possess altered expression profiles, allowing for the detachment of neutrophils from EC and indicating that potential reverse migration mechanisms may occur (Bordon, 2011). The reverse chemotaxis of neutrophils from inflammatory sites was also demonstrated *in vivo*, (Mathias *et al*, 2006). However, it remains unclear whether neutrophils are still functional after reverse migration, or whether their migration is merely directed towards the place of final clearance. In this context, neutrophils with altered expression profiles were localised in the bone marrow, a potential location for final neutrophil clearance (Furze and Rankin, 2008, Suratt *et al*, 2001).

1.2.2.2. Alterations of Neutrophil Lifespan in COPD.

A body of evidence supports a potential alteration in neutrophil apoptosis in COPD. Peripheral blood neutrophil apoptosis in COPD patients was significantly delayed in a temporal manner in stable-state (Zhang *et al.*, 2012) and during exacerbations (Pletz *et al.*, 2004; Schmidt-Ioanas *et al.*, 2006; Juss *et al.*, 2013). Likewise, sputum neutrophil apoptosis was significantly delayed in stable COPD patients by over 20 % (Brown *et al.*, 2009). In a different study, no changes in constitutive apoptosis were detected in circulating neutrophils, but altered surface cytokine

expression in COPD patient neutrophils (increased CD11b and decreased CD62L expression), indicated that peripheral blood neutrophils from COPD patients were already activated before their recruitment to the inflammatory site (Noguera *et al.*, 2004). This may be consistent with the reported systemic cytokine expression in COPD (Moermans *et al.*, 2011; Biffl *et al.*, 1996) that has the potential to contribute to cellular activation and survival. Moreover, inflammatory neutrophil activation may result in pro-inflammatory cytokine release, which can further prolong their lifespan in the lung. Although systemic cytokines expression (Moermans *et al.*, 2011; Biffl *et al.*, 1996) may be present in steady state, it is well accepted that cytokine expression is increased during exacerbations (Wedzicha *et al.*, 2000), and their effect on neutrophil survival may be greater in this scenario. Despite the influence of steroid therapy on inflammation and neutrophil apoptosis (Sin *et al.*, 2004; Zhang *et al.*, 2001), the delay in neutrophil apoptosis was not correlated with cytokine levels (i.e. IL-6, CXCL8 or TNFα levels), nor smoking habits, corticosteroid treatment or state of hypoxemia (Schmidt-Ioanas *et al.*, 2006). Of note, the authors concluded that most "non-smokers" involved in the study stopped smoking only days before the onset of the exacerbation, which may contribute to the lacking difference in neutrophil apoptosis between smokers and non-smokers in their study. It was previously found that alterations in gene expression in smokers took 8 weeks to return to normal (Morozumi *et al.*, 2004), thus indicating that the influence of smoking habits on the outcome of the study (Schmidt-Ioanas *et al.*, 2006) may have even been underestimated.

Taken together, these studies may indicate that the apoptotic process in neutrophils in COPD is dysregulated, where circulating neutrophils have apoptosis levels comparable to healthy controls, but systemic inflammation may increase the susceptibility to neutrophil activation and prime recruited neutrophils. Pro-survival mediators in the inflammatory lung environment, i.e. PGE2, cigarette smoke, LPS and medications, such as PDE inhibitors may then extend neutrophil lifespan. Additional to the reported impairment of macrophage efferocytosis and PS cleavage in susceptible patients, neutrophilia may develop as a functional consequence of increased survival with likewise decreased recognition and clearance.

1.2.2.3. Molecular Regulation of Neutrophil Survival by cAMP/PKA Signalling.

A central intracellular mediator of survival in neutrophils is cyclic adenosine monophosphate (cAMP), which activates the serine threonine kinase PKA (Rossi *et al.*, 1995). PKA is involved in cytoskeleton remodelling, ER to Golgi transport, glycogenolytic cascade, transmission of nerve impulses and muscle contraction. Moreover, PKA plays important roles in the regulation of the cell cycle through the anaphase promoting complex APC and apoptosis, through mitochondrial proteins such as Bad. Activation of gene transcription through CREB further expands its myriad of roles.

cAMP

In COPD, infection with *Pseudomonas aeruginosa* decreases intracellular cAMP and induces neutrophil apoptosis (Usher *et al.*, 2002). Interestingly, extracellular ATP levels were previously proposed as biomarkers for neutrophil-mediated airway inflammation (Esther *et al.*, 2008). cAMP is involved in a variety of pathways, ranging from the cell growth pathway mTOR signalling to exchange proteins activated by cAMP (Epac), cytokine producing p38 signalling, and cell survival in the p7086K pathway, with pro and antiapoptotic effects in neutrophils (Insel *et al.*, 2012, Martin *et al.*, 2001). However, the predominant downstream effectors of cAMP are PKA, Epac and cAMP nucleotide-gated (CNG) membrane channels. cAMP is transformed from ATP by adenylyl cyclase (AC; Sunahara *et al.*, 1996), which in turn is known to be activated through the binding of dissociated subunits of guanosine triphosphate (GTP)-binding proteins, induced by extracellular binding to G protein coupled transmembrane receptors (Strathmann, Simon, 1991). Furthermore, cAMP catalyses the opening of CNG channels, enabling the influx of sodium or calcium ions into the cell, linking it to calmodulin-dependent signalling. Moreover, Epac undergo conformational changes upon binding to cAMP, and then in turn activate the GTPases Rap-1/-2, which can inhibit the MAPK pathway-related GTPase Ras (Bichet, 2006).

PKA

PKA is present in the form of a hetero-tetramer, which is comprised of a dimer of regulatory and catalytic subunits. Binding of cAMP to two distinct sites on the regulatory subunits (Chochung *et al.*, 1989) activates PKA and releases the active catalytic subunits, which become further modulated by phosphorylation. The downstream effectors activated by PKA are as diverse, as its isoforms (regulatory subunits: RIα, RIβ, RIIα, RIIβ; catalytic: Cα, Cβ, Cγ) and are subject to extensive research (Insel *et al.*, 2012). The distinct isoforms are thought to react to different stimuli and elicit diverse responses with distinct affinities to cAMP binding (Skalhegg, Tasken, 2000). Functions of PKA are cell type specific, as PKA mRNA is widely expressed, but its localisation and composition varies (Griffiths *et al.*, 1990, Nigg *et al.*, 1985), as well as its activation in distinct cellular compartments through the varying availability of cAMP (Zaccolo, Pozzan, 2002). PKA has profound antiapoptotic effects in neutrophils (Vaughan *et al.*, 2007) and induction of apoptosis in neutrophils has been described to be mediated by a caspase-dependent mechanism, inhibiting pro-survival PKA/PI3K/Akt pathways (Sousa *et al.*, 2010), overall resulting in the resolution of inflammation. Pro-apoptotic effects are mediated through activation of transcription by phosphorylation of the cAMP-responsive element binding protein (CREB) in the nucleus, influencing gene-expression (Montminy *et al.*, 1990), and phosphorylation of NF-κB. PKA also inhibits 14-3-3, linking it to Bcl-2 signalling, as it prevents the promotion of cell survival through phosphorylation of Bad. Interference with the

JNK pathway exerts pro-and anti- apoptotic effects, by inhibition of Rho phosphorylation (Liu, Lin, 2005), as well as its interaction with extracellular-regulated kinase (ERK) signalling by inhibition of phosphorylation of Rho and activation of ERK, which is important in the regulation of various proliferative effects (Juan *et al.*, 2010). A molecular mechanism of the prosurvival effects of PKA has been shown by the Sabroe/Whyte groups, who found the ATP-receptor P2Y11 to be implicated in translation of extracellular ATP to intracellular cAMP signals (unpublished data), which might then through PKA support neutrophil survival (Vaughan *et al.*, 2007). Interestingly, ATP is often released by cells damaged in infection and inflammation (Vaughan *et al.*, 2007), implicating this P2Y receptor in neutrophil survival in infection. Universally, PKA is highly susceptible to changes in the levels of the second messenger cAMP, which is its sole substrate.

1.2.2.4. Therapeutical Strategies in COPD.

COPD still remains a major cause of death and current treatments are inadequate, as they may alleviate some of the symptoms, such as bronchoconstriction but are unable to reverse the disease progression. Treatment failure can have various causes, such as a failure to recognise the most suitable therapeutic target or disease characteristic (Sabroe *et al.*, 2007). Despite the cytotoxic potential of neutrophils in COPD, neutrophilia has been particularly neglected in COPD therapy, and commonly used therapeutic strategies, such as β adrenoreceptor agonists and corticosteroids may prolong neutrophil lifespan and thus aggravate their persistence in the lung. A broad view approach to COPD treatment thus requires taking the overall contribution of neutrophils to disease severity into account, additional to their role in inflammatory gene expression. As elaborated in the previous chapter, a number of factors can increase the presence of neutrophils in the COPD lung and here, the contribution of current therapeutic options in the context of neutrophilia will be explored.

Current Treatments and Effect on Neutrophils.

Combination treatment with β adrenoreceptor agonists and corticosteroids is a mainstay of the current therapeutic options in the treatment of COPD (Rabe *et al.*, 2007; Singh *et al.*, 2008). The use of both drugs in COPD is controversely discussed due to their partly limited efficacy and the induction of non-specific side-effects (Meijer *et al.*, 2013; Salpeter *et al.*, 2004; Cazzola *et al.*, 1998; Calverley *et al.*, 2007). Additionally, muscarinic receptor antagonists are a common therapeutic option in COPD (Montuschi et al., 2013). Here, the effects of these drug classes on inflammation and neutrophil lifespan regulation will be briefly reviewed.

β adrenoreceptor agonists

β adrenoreceptor agonists are available in two forms: short-acting and long-acting. Clinically, they are used as bronchodilators that increase airflow by relaxing airway smooth muscle cells (Di Marco *et al.*, 2003; O'Donnell *et al.*, 2004). Bronchodilators may temporarily alleviate the

symptoms of CODP, in particular during exacerbations (Rossi *et al.*, 2008; Appleton *et al.*, 2006). However, bronchodilators, including β adrenoreceptor are ineffective at the reversal of airflow obstruction and NE secretion, as bronchoconstriction is not a driver of the disease progression (Meijer *et al.*, 2013; Sabroe *et al.*, 2007; Barnett *et al.*, 1997). Moreover, they have been associated with an increased risk of cardiovascular complications (Cazzola *et al.*, 1998), and β adrenoreceptor agonists did not reduce the overall mortality in COPD (Kliber *et al.*, 2010).

In fact, bronchodilators work by increasing the levels of cAMP (Barnes, 1993), which is a potent pro-survival mediator in neutrophils (Rossi *et al.*, 1995). In fact, it was found that neutrophil survival was enhanced by treatment with corticosteroids and that this could be increased by simultaneous treatment with β adrenoreceptor agonists (Pertunnen *et al.*, 2008), indicating that the current therapeutic options in COPD may further increase neutrophilia in the condition.

Corticosteroids

Corticosteroids are a strategy of choice in the treatment of asthma and are often prescribed to COPD patients (Meijer *et al.*, 2013). However, their efficacy in the treatment of COPD is limited to a subset of COPD patients with increased eosinophilic infiltration (Fujimoto *et al.*, 1999; Barnes, 2006; Jen *et al.*, 2012; Barnes, 2013) and may help to reduce the occurrence of exacerbations (Calverley *et al.*, 2007; Sabroe *et al.*, 2013). However, it was recently determined that glucocorticoid treatment may have immunomodulatory properties and may predispose patients to develop secondary lung infections (Sabroe *et al.*, 2013). In particular, steroid-treatment does not target neutrophilic inflammation (Bianchi *et al.*, 2006; Haslett, 1997; Barnes, 2013), nor considerably reduces neutrophil or macrophage numbers (Jen *et al.*, 2012), whereas it greatly reduces T cell presence. Corticosteroids may reduce systemic inflammation (Sin *et al.*, 2004), but are ineffective at the suppression of inflammatory mediator secretion, as the expression of the metalloprotease MMP-9, NE and various cytokines, such as CXCL8 and TNFα is not altered by steroid treatment (Keatings *et al.*, 1997; Culpitt *et al.*, 1999; Loppow *et al.*, 2001) with only GM-CSF secretion being suppressed (Barnes, 2004). The lack of responsiveness to corticosteroids may be a unique feature of COPD, as the responsiveness of alveolar macrophages in COPD patients to corticosteroids is reduced (Culpitt *et al.*, 2003). Moreover, corticosteroids inhibit LPS-induced NE release only in neutrophils from non-smokers (Barnes, 2003; Meijer *et al.*, 2013).

In fact, the use of corticosteroids may be contraindicated in COPD patients with neutrophilia, but no concomitant asthma, as steroid treatment was consistently shown to delay neutrophil apoptosis (Chang *et al.*, 2004; Saffar *et al.*, 2011; Marwick *et al.*, 2013), potentially increasing the severity of disease progression through the augmentation of neutrophilia. Steroids inhibit apoptosis *in vitro* (Zhang *et al.*, 2001; Balaban *et al.*, 2005) and systemic steroid treatment

during exacerbations or pulmonary infections coinciding with increased PMN survival (Zhang *et al.*, 2001; Pletz *et al.*, 2004; Strassburg *et al.*, 2010). The use of corticosteroids is controversely discussed due to the potential risks associated with this type of medication (Sabroe *et al.*, 2013; Babu *et al.*, 2014). Thus, alternative or complimentary therapeutic strategies are urgently required to combat neutrophilia in COPD.

Muscarinic Receptor Antagonists

Several antimuscarinic drugs, such as aclidinium bromide, ipratropium bromide, oxitropium bromide and tiotropium bromide are used in COPD therapy (Montuschi *et al.*, 2013). The low efficacy of these compounds in COPD therapy and controversial clinical outcomes, novel antimuscarinic drugs are still under development to prolong the short action of these compounds in the lung (Moulton, Fryer, 2011). Muscarinic receptor antagonists block the activation of the muscarinic actetylcholine receptors M1, M2, M3 and M4 and the presence of M1, M2 and M3 receptors on neutrophils in COPD was demonstrated by Profita *et al.* (2005). The authors found that muscarinic receptors were implicated in LTB4 release, which may potentially lead to decreased neutrophil activation in COPD therapy with muscarinic receptor antagonists.

Although the role of muscarinic receptor antagonists in neutrophil survival is not clear, it has been shown that carbachol, an activator of M1 and M3 receptors, potently induces Cox-2 expression in airway smooth muscle cells (Kanefsky *et al.*, 2006). Potentially, this may indirectly increase neutrophil survival through the induction of PGE2 secretion. This may be consistent with the reported reduction in neutrophil numbers in BAL through treatment with the non-selective muscarinic receptor antagonist tiotropium (Wollin, Pieper, 2010). However, tiotropium also blocked M3-receptor mediated neutrophil chemotaxis (Kurai *et al.*, 2012), indicating that the neutrophil reducing function of the compound may also rely on its interference with cell migration. This is further supported by the findings of Profita *et al.* (2005), who found increased M3-dependent chemotactic abilities in COPD neutrophils.

Novel Therapies and Effect on Neutrophils.

Various novel therapeutic strategies have been explored in the treatment of COPD experimentally and in drug trials, and the regulation of neutrophil lifespan is of major clinical interest (Bianchi *et al.*, 2006). In fact, increasing neutrophil apoptosis and thus reducing their numbers in the lung has resulted in a potent anti-inflammatory response in *in vitro* and *in vivo* models of COPD (Sydlik *et al.*, 2013). In particular, anti-neutrophilic strategies may be unfavourable in early, but highly valuable in advanced stages of the disease, where the cytoxic effects of neutrophils outweigh their efficacy in driving the immune response (Bianchi *et al.*, 2006).

Adenosine A2A receptor agonists

The adenosine A2A receptor is involved in the cAMP-dependent activation of PKA signalling (Barletta *et al.*, 2012), and is expressed on neutrophils (Pliyev *et al.*, 2014). The expression of A2A in the COPD lung parenchyma is increased (Varani *et al.*, 2006), implicating cAMP/PKA signalling in COPD disease pathology. Therapeutic targeting of A2A receptors showed encouraging effects on smooth muscle cell relaxation and inflammatory cell recruitment, but a mixed response on inflammation (Xu *et al.*, 2011; Yang *et al.*, 2014; Philis, 2004; Bonneau *et al.*, 2006; Polosa, Blackburn, 2009). In neutrophils, activation of the pro-survival receptor A2A promotes the production of Cox-2 derived PGE2, potentially encouraging the self-sustained characteristics of the disease. A2A agonism also inhibits various cellular functions in neutrophils, such as the inflammatory cytokine release, NE and ROS production, as well as degranulation (Barletta *et al.*, 2012). A therapeutic intervention with A2A receptor agonists would thus require combinational treatment with a second drug to effectively control the inflammatory response. As A2A receptor agonism promotes neutrophil survival, it may be hypothesized that such a drug may target neutrophil survival. Current clinical trials have revealed cardiovascular side-effects of the drugs and no significant effects on disease pathology (Polosa, Blackburn, 2009; Beeh, Beier, 2006)

PDE4 inhibitors

Phosphodiesterases (PDE) catalyse that degradation of cAMP (Chang *et al.*, 2000) and PDE4 is the main subtype present in neutrophils (Bäumer *et al.*, 2007). PDE inhibition was shown to be of value in the treatment of extensive airflow limitation in COPD, as it induces the relaxation of airway smooth muscle cells (Beghe *et al.*, 2013; Mehats *et al.*, 2003), and are thus considered to be a potential therapeutic strategy in COPD (Garnock-Jones, 2015). PDE4-induced termination of the cAMP/PKA signal induces the expression of pro-inflammatory mediators, such as TNFα, CXCL8 and IFNγ (Jimenez *et al.*, 2001; Au *et al.*, 1998; Sheibanie *et al.*, 2004). PDE4 inhibitors suppressed the CXCL8 generation in activated neutrophils (Au *et al.*, 1998) and this was enhanced by PGE2 treatment, suggesting that some anti-inflammatory effects of PGE2 on cytokine expression may be mediated via PKA signalling. Evidence for a dual role of cAMP/PKA signalling in inflammation exists, as PDE4 inhibition also increases the anti-inflammatory cytokine IL-10 (Oger *et al.*, 2005).

Cox inhibitors

Cyclooxygenases (Cox) mediate prostaglandin secretion. Cox2 in particular, has been linked to the LPS-induced secretion of PGE2. Due to the role of PGE2 in inflammatory gene expression, extension of neutrophil survival and its effects on mucus secretion (Meja *et al.*, 1997; Rossi *et al.*, 1995; Borchers *et al.*, 1999), inhibition of Cox was regarded as a potential therapeutic strategy. In fact, a non-selective Cox inhibitor reduced mucus hypersecretion in COPD (Tamaoki *et al.*, 1992), and Cox2 inhibitors showed anti-inflammatory potential for the

treatment of COPD (Newton *et al.*, 1997). Nevertheless, the LPS-induced expression of the inflammatory marker TNFα by monocytes was prevented by PGE2, but became even more pronounced upon treatment with a non-selective Cox inhibitor (Ulcar *et al.*, 2004). Additionally, the development of peptic ulcers and severe cardiovascular side-effects with these drugs were reported (Mukherjee *et al.*, 2001; Hippisley-Cox, Coupland, 2005) and thus their experimental development halted.

Putative Strategies in COPD Therapy.

To conclude, both inhibition, as well as activation of PKA in inflammation has been shown to have therapeutic potential, supporting a dual role of PKA signalling in COPD pathology. Activation of PKA may induce anti-inflammatory cytokine expression, but likewise prolong the lifespan of immune cells. Once activated, immune cells such as neutrophils may then contribute to tissue damage in the condition and further increase the susceptibility to infection, overall exacerbating the disease severity. The pro-survival molecules PGE2 and LPS can have both anti- and pro-inflammatory functions, which may cause the unsatisfactory outcomes of many novel therapeutical strategies. Thus, their role in the molecular control of survival signalling in neutrophils requires a further detailed examination, and a common survival-specific downstream target may prove to be a magic bullet in the treatment of COPD.

1.2.3. Neutrophil Models

The most prominent feature of neutrophil biology is their constitutive apoptosis, making them unique amongst most other cells. The short lifespan of neutrophils limits experimental potential. A comprehensive characterisation of pathways underlying constitutive death and survival is challenging, as neutrophils are not only short-lived, but also post-mitotic and hence poorly genetically modifiable. Modulating the immune system through the alteration of neutrophil lifespan has been long proposed to be a valuable target in diseases. Therefore, introducing genetic modifications in model organisms from which genetically modified neutrophils can be obtained, has been of major interest to immunological research. As apoptosis is highly conserved in morphology and pathway events throughout most species, (i.e. *Mus Musculus*, *Danio rerio*, *Drosophila melanogaster*, *Caenorhabditis elegans*), including plants, apoptosis pathways can be successfully researched in species different to our own, whilst being aware that differences might be present.

Immunology in the last decades has greatly relied on the use of genetically modified mice. Mouse neutrophil models used in this study were thereby derived from BALB/c mice, which can be used as an *in vivo* model in immunology, as this inbred strain possesses an intact immune system in contrast to most other laboratory mice strains. However, only limited amounts of

neutrophils can be obtained from the blood, bone marrow or the peritoneal cavity of genetically modified mice.

1.2.3.1. Murine Model.

BALB/c is an established inbred immunocompetent laboratory strain, which displays good immune responses to most stimuli. However, neutrophils from BALB/c mice show immune responses to stimuli, such as killed bacteria and thioglycolate broth, which are consistently lower in comparison to immune responses of C57BL/10 mice (Marley *et al.*, 1994). More recently, it has been proposed that laboratory strains, raised in disease-free conditions, might not hold a representable immune system in contrast to wild-living mice, as these are in contrast exposed to pathogens that function to prime immune cells (Boysen *et al.*, 2011).

However, there are also known differences between the immune system of humans and mice, which is not surprising, as evolutionary progenitors of the species (Lamkanfi *et al.*, 2002), were more likely to be exposed to different immunological tasks in their respective ecological niches, which is further evidenced by the higher occurrence of differences in adaptive immunity between the two species. Therefore, it is crucial for the prevention of translational failures to be aware of existing differences. The most obvious difference between humans and mice is the distribution of neutrophils in the blood. While neutrophils are abundant in human blood, in comparison there are generally more lymphocytes present in wild type murine blood (Doeing *et al.*, 2003).

Moreover, haematopoietic stem cells differ in expression patterns (see chapter 1.2.1.1). Mice derive defensins from Paneth cells in the small intestine, while in humans, neutrophils are able to directly express defensins (Risso, 2000). However, mice possess more different kinds of defensins than humans, highlighting the diverse immunological challenges due to different ecological niches and the adaptation to these. This is evidenced by the differential TLR expression amongst the two species. While TLR10 is expressed in humans, the receptors TLR11, TLR12 and TLR13 are more predominantly expressed in mice (Mestas, Hughes, 2004). In Balb/c mice, lower expression of TLR4 receptors has been found in various immune cells, coinciding with a lower responsiveness to TLR4 stimulation (Oliveira *et al.*, 2014a; Tsukamoto *et al.*, 2013). Furthermore, the expression on granulocytes differs, as CD33 is for instance expressed on all murine granulocytes, but in humans expression is limited to monocytes (Brinkman-Vand der Linden *et al.*, 2003). The response to fMLP has a higher affinity in humans, due to structural aberrations of the structural domains in mice (Gao, Murphy, 1993). Absent in mice and present in humans are furthermore Caspase 10 (Tibbetts *et al.*, 2003), ICAM3 (Geijtenbeek *et al.*, 2000). They also differ in chemokine expression, most notably, CXCL8 and its receptor CXCR1 is absent in mice (Zlotnik, Yoshie, 2000, Olson, Ley, 2002).

1.2.3.2. Estrogen-regulated Hoxb8 Neutrophil Progenitors (mCMP) and derived Neutrophils (mNØ)

A recent approach to enable the generation of large quantities of genetically modified neutrophils from knockout mice has been described (Wang *et al.*, 2006). This approach is based on the discovery that Class I Homeobox (Hox) Transcription Factors are highly expressed in $CD34^-$ Haematopoietic stem cells (HSC) and myeloid lineage committed progenitors (LCP), while they become downregulated with ongoing differentiation, as marked by the expression of CD34 (Lawrence *et al.*, 1997, Pineault *et al.*, 2002). Moreover, expression of Hoxb8 has also been shown to directly arrest the differentiation of myeloid progenitors (Fujino *et al.*, 2001, Knoepfler *et al.*, 2001), and thereby allows the expansion of myeloid progenitors via expression of Hox oncoproteins. In this model, BALB/c lineage-negative progenitors are factor-dependently immortalised through the expression of a conditional form of the Hoxb8 transcription factor with an estrogen receptor (ER)-binding domain fusion. Upon inactivation of the conditional Hoxb8 and supplementation with neutrophil lineage specific growth factors, mCMP terminally differentiated into neutrophils (mNØ), which had exited the cell cycle after the third day of differentiation and displayed typical immune responses, as well as not becoming aneuploid due to differentiation, nor significantly in the time of long term culture (Wang *et al.*, 2006).

To evaluate differences between peripheral blood neutrophils and neutrophils derived from mCMP, marker expression profiles were characterised by Wang *et al.* (2006). It was highlighted that neutrophils derived from mCMP showed upregulated NADPH oxidases. Neutrophil gene markers were upregulated (GR-1, CD177 neutrophil marker of unknown function, IL8rb, IL17r, CD73), as were myeloid differentiation markers (CD14, Itgam, TLR2, IL1β) and general myeloid gene markers (TLR2, CD14, CD11b, Csf2rα, Mac-1 and Mannose receptor). Downregulation of differentiation-related genes and transcription factors, stem cell receptor and transcription factor gene markers (CD34, Meis1; specific haematopoietic), as well as promyelocyte genes and gene markers responsible for cell division CycB1 (G2/M progression), nucleotide biosynthesis, DNA replication, RNA maturation, Ribosome biogenesis and cell cycle further supported a differentiation to functionally mature neutrophils (Wang *et al.*, 2006). Marker expression profiles are comparable to the expected profiles for primary human neutrophils (Wang *et al.*, 2006) and resemble morphologically mature murine neutrophils (McDonald *et al.*, 2011). The functionality of mNØ in the research of apoptosis was previously shown (Kirschnek *et al.*, 2011).

1.3. The Inflammatory Mediator PGE2 and Its Pro-Survival Signalling Pathways in Neutrophils.

PGE2 is a central regulator of inflammation in the context of COPD that is thought to elicit both pro- and anti-inflammatory responses through the differential expression of prostaglandin receptor subtypes. The prostaglandin is most notably secreted by fibroblasts, epithelial cells and further immune cells, such as monocytes (Saalbach et al., 2015; Schmidt et al., 2011; Fogel-Petrovic et al., 2004). PGE2 was previously noted to be a crucial mediator of airway inflammation in the presence of cigarette smoke (Lin *et al.*, 2010). Moreover, its expression becomes significantly enhanced in LPS-activated monocytes and macrophages in the course of the immune response, where LPS induces a 300 fold increase in PGE2 release compared to a medium control (Ikegami *et al.*, 2001). A correlation between neutrophil numbers, endothelial cell adhesion and PGE2 levels in COPD smokers exist, whereby PGE2 secretion is promoted by cigarette smoke extract treatment, potentially contributing to neutrophilia in COPD (Profita *et al.*, 2010). The pleiotropic effects of PGE2 vary in different cell types and are thought to depend on differential receptor subtype expression and the amount of PGE2 present in the microenvironment of the cell, where the high-affinity receptors EP3 and EP4 may become activated by lower PGE2 concentrations (Konger *et al.*, 2005). Moreover, the distinct receptor subtypes activate divergent signalling pathways.

In neutrophils, PGE2 interferes with fMLP-induced signalling (Burelout *et al.*, 2007; Kanamori *et al.*, 1997; Talpain *et al.*, 1995; Wheeldon, Vardey *et al.*, 1993; Armstrong, 1995; Ham *et al.*, 1983) and prevents LTB4 release following phagocytosis (Wheeldon, Vardey, 1993). PGE2 itself is a potent pro-survival molecule for neutrophils that is increased in COPD smokers (Rossi *et al.*, 1995; Ottonello *et al.*, 1998; Montuschi *et al.*, 2003; Kato *et al.*, 2006; Dick *et al.*, 2009; Profita *et al.*, 2010), and has been shown to increase intracellular cAMP (Rossi *et al.*, 1995). However, it may also induce apoptosis in cytotoxic $CD8^+$ T lymphocytes (Chen *et al.*, 2015). In steady-state, alveolar macrophages express Cox2 (Jiang *et al.*, 2003), the expression of which is increased in COPD and correlates with increased PGE2 levels (Taha *et al.*, 2000; Profita *et al.*, 2010).

PGE2 may have both pro- and anti-inflammatory roles in COPD. PGE2 is linked to the development of classical inflammatory hallmarks, such as redness, pain, swelling and also fever (Funk, 2001). Moreover, PGE2 acts as a vasodilator, which enables the increased flow of blood, and thus the influx of immune cells to the inflamed tissue (Funk, 2001) and is also involved in the induction of mucus secretion (Borchers *et al.*, 1999). Further pro-inflammatory functions are exerted through the expression of cytokines in neutrophils and the inhibition of monocytic release of pro-inflammatory cytokines (Meja *et al.*, 1997). Moreover, PGE2 has been shown to act as a bronchodilator (Pavord, Tattersfield, 1995), but its metabolic product PGF2α, which is

also increased in COPD, may have the opposite effect (Montuschi *et al.*, 2003; Nichol *et al.*, 1990). As an instigator of inflammatory cytokine expression, the effect of PGE2 ranges from supporting TNFα-induced IL-6 secretion (Kunisch *et al.*, 2009) to the inhibition of anti-inflammatory IFNγ and IL-2 expression in PBMC (Pripp, Stanisic, 2014; Corrigan *et al.*, 2012). It interferes with LPS-induced inflammatory cytokine secretion of the pro-inflammatory cytokines IL-6 and TNFα (Pripp, Stanisic, 2014). In particular, PGE2 augments LPS-mediated IL-6, but blocks TNFα secretion in murine peritoneal neutrophils (Yamane *et al.*, 2000). Moreover, the increased secretion of PGE2 in COPD fibroblasts has been proven to halt proliferation, as well as increasing pro-inflammatory cytokine expression (Dagouassat *et al.*, 2013). PGE2 mediates the macrophage-induced inflammation in septic shock (Xiang *et al.*, 2013) and can also account for the promotion of TH17 cell differentiation (Boniface *et al.*, 2009; Chizzolini *et al.*, 2008; Napolitani *et al.*, 2009).

The anti-inflammatory effects of PGE2 may be exerted by the inhibition of pro-inflammatory cytokine secretion, such as IFNγ, IL-2 and TNFα in macrophages (Pripp, Stanisic, 2004; Corrigan *et al.*, 2012; Meja *et al.*, 1997). LPS transcriptionally activated the expression of the pro-inflammatory cytokines IL-1, CXCL8 and cytokines with dual properties in inflammation, such as TNFα and IL-6 (Cohen, 2002) in COPD. Neutrophil treatment with PGE2 suppressed LPS induced TNFα and enhanced IL-6 secretion (Yamane *et al.*, 2000). Immunosuppressive properties of PGE2 have so far been proposed by several groups (van der Pouw *et al.*, 1995; Ferreri *et al.*, 1992).

The contrasting role of PGE2 in inflammation may also be exerted through the products of protein metabolism and the selective affinity of these products to the distinct protein receptors. So has, for example PGD2 been shown to have a dual effect on functional responses, such as the induction of the respiratory burst and apoptosis at nanomolar to micromolar concentrations respectively (Sandig *et al.*, 2007). Moreover, PGE2 acts as a bronchodilator in human airways (Pavord and Tattersfield, 1995), whereas its degradation product PGF2α is a bronchoconstrictor that like PGE2 is increased in COPD patients (Nichol *et al.*, 1990; Montuschi *et al.*, 2003; Profita *et al.*, 2010). The distinct PGE2 receptor subtypes have differential roles in this context. PGE2 mediated TNFα suppression was mediated by the EP4 receptor (and partially by the EP2 receptor), while IL-6 enhancement was mediated by EP2 only. However, also interactions of LPS and PGE2 have been reported to play a role in pro-survival cytokine generation in COPD. LPS primes anti-inflammatory and pro-survival G-CSF production, and PGE2 augmented this (Sugimoto *et al.*, 2005), as well as IL10 production. PGE2 suppresses LPS-stimulated IFNβ production (Xu *et al.*, 2008) and there is also an inhibitory effect of PGE2 and cAMP on LPS-induced release of TNFα, and IL-6 production. Moreover, autocrine feedback mechanisms of LPS signalling by LPS-induced PGE2 production were previously proposed (Strassmann *et al.*, 1994; Benninghoff *et al.*, 1988).

Thus, PGE2 has roles in the promotion and resolution of inflammation in inflammation and COPD. As it has the potential to perpetuate its own synthesis, by increasing Cox-2 expression, it may contribute to the self-sustained characteristics of chronic inflammation in COPD. Due to the rapid effects of prostaglandin production that are largely independent of translation and transcription, they can readily increase the expression of early response genes in inflammation, such as nuclear receptors. Therefore, a selective target downstream target of PGE2 involved in the transmission of its pro-inflammatory effects, such as increasing neutrophil survival, would thus be an interesting therapeutical target in COPD.

1.3.1. Biosynthesis and metabolism of PGE2

1.3.1.1. PGE2 Biosynthesis.

Arachidonic Acid (AA) is the metabolic precursor of prostaglandin synthesis, the enzymatic metabolism of which can lead to a rapid increase in prostaglandin levels, as it is largely independent of transcriptional and translational mechanisms. AA is oxidized by Cyclooxygenase 1 (Cox-1) or Cyclooxygenase 2 (Cox-2) to PGH2 (O'Neill *et al.*, 1994; Marnett *et al.*, 1999; **Fig. 3**). PGH2 synthesis can occur either directly or in a two-step process with PGG2 being an intermediate reaction product in a process termed cyclooxygenase and peroxidase reaction respectively (Garavito *et al.*, 2002; Chubb *et al.*, 2006). Subsequently, PGH2 is metabolically transformed to the major bioactive prostaglandins PGD2, PGE1; PGE2, PGF2α or PGI2 by their respective synthase (Hayashi *et al.*, 1989; Thoren *et al.*, 2003; Murakami *et al.*, 2000; Murakami *et al.*, 2003; Hayashi *et al.*, 1989; Lim *et al.*, 2000), such as PGES in PGE2 production (Jakobsson *et al.*, 1999). The expression of these prostaglandins varies in different cell types. Interestingly, whereas Cox-1 is ubiquitously and constitutively expressed, Cox-2 expression is positively regulated by its main metabolite PGE2, thereby perpetuating PGE2 expression (Dubois *et al.*, 1998; Brock *et al.*, 1999).

In the course of inflammation, the expression of prostaglandins increases in cells such as mast cells (Tilley *et al.*, 2001), whereas the LPS-induced cell activation in cells such as macrophages, promotes a shift from TXA2 to PGE2 secretion, with an over 200 fold increase in PGE2 expression (Ikegami *et al.*, 2001). Thus, prostaglandins have been proposed as inflammatory markers. Non-steroid anti-inflammatory drugs (NSAIDs), such as ibuprofen (Cryer *et al.*, 1998) are known to non-selectively inhibit Cox enzymes and prostaglandin synthesis, whereas aspirin or diclofenac are more selective towards Cox-1 or Cox-2 respectively. Their common action on inflammation despite the structural differences of these compounds has long supported the importance of prostaglandins in inflammation (Vane, 1971). As Cox-2 is primarily overexpressed in the context of inflammation (Cryer *et al.*, 1998),

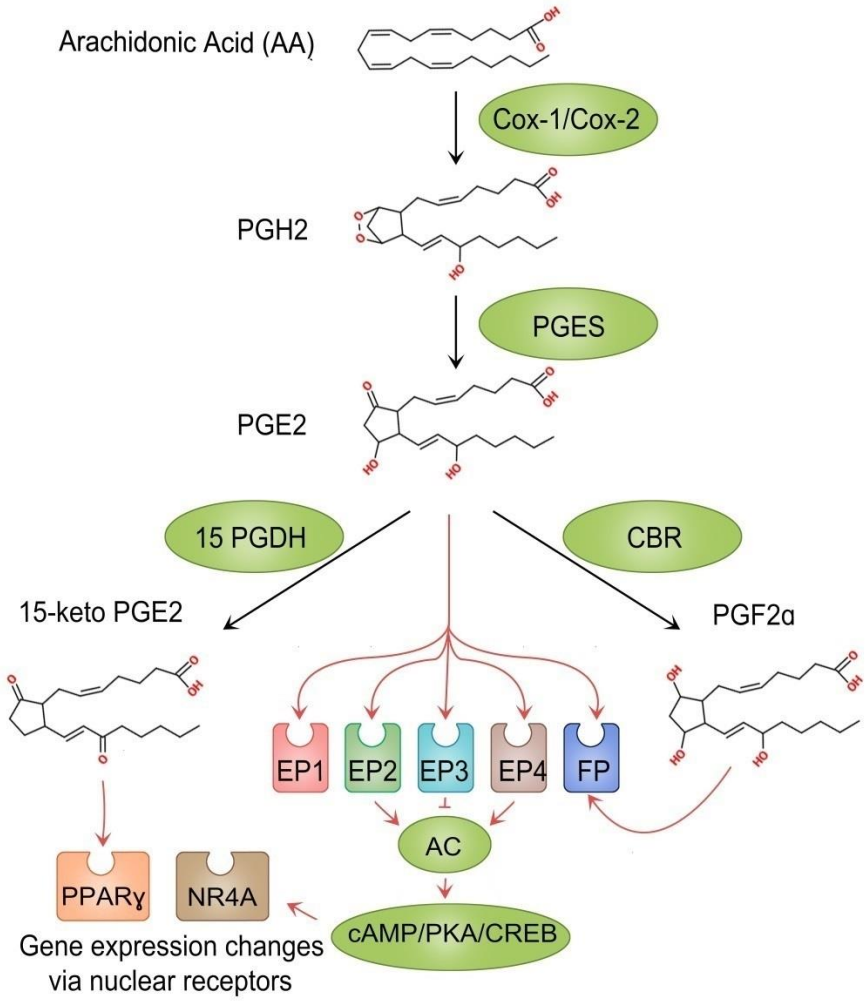


Figure 3: ***Metabolism and Receptor Engagement of PGE2 and its Degradation Products.*** Arachidonic Acid (AA) metabolism initiates by the NSAID-inhibitable oxidation of AA to PGH2 by the action of Cox-1 or Cox-2. Prostaglandin E synthase (PGES) then mediates the transformation of PGH2 to PGE2, which can activate cAMP/PKA/CREB signalling and modulate gene expression via NR4A receptors and through the engagement of the prostaglandin receptors EP2 and EP4. PGE2 itself can be reduced to the PPARγ agonist 15-keto PGE2 by 15-hydroxyprostaglandin dehydrogenase (PGDH) or to the FP receptor agonist PGF2α by carbonyl reductases (CBR).

selective Cox-2 inhibitors, such as celecoxib, rofecoxib and etoricoxib were employed to control inflammation (Mardini, FitzGerald, 2001); however, these displayed additional risks for unexpected cardiovascular complications (Friedewald *et al.*, 2010), as opposed to the increased gastrointestinal side-effects of Cox-1 inhibitors (Cryer *et al.*, 1998). Thus, diverse studies now support an early role of Cox-1-derived prostaglandins, whereas a dual role of subsequent induction of Cox-2 expression has been reported (Smyth *et al.*, 2009; Langenbach *et al.*, 1999; Gilroy *et al.*, 1999; Wallace *et al.*, 2000; Gavett *et al.*, 1999).

1.3.1.2. Metabolic Products of PGE2.

PGE2 is rapidly metabolized *in vivo* by the action of 15-PGDH (Tai *et al.*, 2002; Watzer *et al.*, 2009; Förstermann, Neufang, 1983; Nishigaki *et al.*, 1996), whereas the presence of albumin determines its stability in an *in vitro* setting (Fitzpatrick *et al.*, 1980). The metabolic products of PGE2 are varied. PGE2 can be directly catalysed to PGF2α by the carbonyl reductases CBR1, CBR2 and CBR3 (Inazu *et al.*, 1992; Nakayama *et al.*, 1986; Nakanishi *et al.*, 1996) or dehydrogenases (Cho *et al.*, 2005). However, PGF2α synthesis can also occur through the formation of the intermediate product 15-oxo-PGE2, mediated by the dehydrogenase 15-PGDH (Cho *et al.*, 2006). Another major metabolic product with functional activity is 15-keto PGE2 (Lu *et al.*, 2013), which is synthesized through the action of 15-PGDH (Schlegel, Greep, 1976). Although 15-keto PGE2 has a significantly lower affinity to EP4 than EP2, further metabolisms are required to reduce the affinity of catabolic products to EP2 (Nishigaki *et al.*, 1996). Through several subsequent modifications, PGE2 can also be transformed to PGB2, which has significantly reduced functional activity at PGE2 receptors (Polet, Levine, 1975).

1.3.2. Activation of PGE2 Signalling Pathways by EP Receptors.

PGE2 mediates its autocrine and paracrine effects by the involvement of four receptor subtypes, namely EP1, EP2, EP3 and EP4 (Konger *et al.*, 2005). EP receptors are G-protein coupled transmembrane protein receptors that are primarily located on the outer plasma membrane of various cell types of internal organs, such as the lung, and in the immune, secretory, muscle, reproductive and nervous systems (Belinsky *et al.*, 2015). Apart from these classical PGE2-related receptors, PGE2 is furthermore known to target the PGF2α receptor FP, albeit with a diminished efficacy.

1.3.2.1. EP1

EP1 receptors are expressed in the plasma membrane and EP1 mRNA is particularly abundant in the cerebellum and the skin. In the immune system, EP1 has been found in whole blood and lymph nodes, but there are no consistent results as to its expression on white blood cells. In particular, its mRNA expression was not detected in neutrophils (Yamane *et al.*, 2000). The intracellular signalling pathways engaged by EP1 agonism target phospholipase C (PLC),

leading to both, the activation of protein kinase C (PKC) and calcium mobilisation through increases in inositol 1,4,5-trisphosphate (IP_3; Konger *et al.*, 2005; Rundhaug *et al.*, 2011). However, EP1 receptors did not elicit functional responses in neutrophils in a variety of assays, such as fMLP-stimulated O_{2^-} generation and phagocytosis-induced LTB4 release (Kanamori *et al.*, 1997; Talpain *et al.*, 1995; Wheeldon, Vardey, 1993).

1.3.2.2. EP2

EP2 is mainly localised in the plasma membrane, but may also be present in the extracellular space (Belinsky *et al.*, 2015), where its role remains to be elucidated. EP2 mRNA is widely distributed throughout the body, and consistently found to be increased by microarray, RNAseq and SAGE assays in the brain, lung, skin and prostate tissues. Basal presence of EP2 protein was particularly pronounced on CD4+ T cells and PBMC. EP2 activation induces a rise in intracellular cAMP levels and subsequent PKA activation through the induction of adenylyl cyclase (AC; Rundhaug *et al.*, 2011). In neutrophils, EP2-mediated signalling prevented secondary effects induced by fMLP, such as calcium influx, O_{2^-} generation and PKA-independent chemotaxis (Burelout *et al.*, 2007; Kanamori *et al.*, 1997; Talpain *et al.*, 1995; Armstrong, 1995). EP2 receptor stimulation is functionally linked to neutrophil immune function by NETosis (Domingo-Gonzalez *et al.*, 2015). EP2-mediated signalling induced the expression of IL-6, and downregulated TNFα expression in murine peritoneal neutrophils (Yamane *et al.*, 2000). Moreover, phagocytosis-induced LTB4 release was inhibited (Wheeldon, Vardey, 1993). A functional loss of EP2 receptors through a mutation in the EP2 receptor gene *PTGER2* has been linked to an increased sensitivity and susceptibility to the development of aspirin-mediated asthma (Jinnai *et al.*, 2004), suggesting that EP2 signalling may be required to limit aspirin-induced side-effects, such as bronchoconstriction due to smooth muscle cell contraction, in the lung. In aspirin sensitive asthma patients, the presence of bronchial mucosal neutrophils was increased, but their gene expression for the EP2 receptor was decreased (Corrigan *et al.*, 2012). Nevertheless, a reduction in cytokine production of IFNγ, IL-2, IL-4 and IL-5 was achieved through EP2 agonism, indicating that the specific impairment of PGE2 signalling in neutrophils contributes to the pathology of the condition.

1.3.2.3. EP3

EP3 is primarily localised in the plasma membrane and the nucleus (Belinsky *et al.*, 2015). EP3 mRNA is often found in the cerebellum, spinal cord, skin and prostate tissues, and EP3 protein, but not mRNA was found in neutrophils (Belinsky *et al.*, 2015; Yamane *et al.*, 2001). EP3 activation provides a counterbalance to EP2 and EP4 activation, as it blocks the activation of AC (Rundhaug *et al.*, 2011) and prevents smooth muscle cell relaxation. In neutrophils, EP3 may contribute to the fMLP-stimulated O_{2^-} generation (Kanamori *et al.*, 1997; Talpain *et al.*, 1995).

1.3.2.4. EP4

EP4 is widely distributed throughout the cell and can be found in the plasma membrane, nucleus and cytosol, but also the extracellular space (Belinsky *et al.*, 2015). EP4 mRNA, like EP2 mRNA can be found in various tissue types, amongst others the whole blood. Most interestingly, EP4 proteins are most abundantly secreted in the cardia, the lung and CD8+ T cells (Belinsky *et al.*, 2015). Much like EP2 receptors, EP4 receptor-initiated signalling pathways involve the stimulation of cAMP and PKA (Rundhaug *et al.*, 2011; Mizuno *et al.*, 2014), and likewise result in the relaxation of smooth muscle cells. EP4 has also roles in the prevention of vascular permeability (Kampitsch *et al.*, 2012). In this context, PGE2 is thought to mediate its effects on the promotion of endothelial barrier function through activation of the EP4 receptor subtype. The EP4 receptor subtype was not linked to fMLP-stimulated O_2 generation (Kanamori *et al.*, 1997; Talpain *et al.*, 1995), but as EP2 increased IL-6 generation, blocked TNFα secretion (Yamane *et al.*, 2000) and was linked to NETosis (Domingo-Gonzalez et al., 2005).

1.3.2.5. Congruencies and Dissimilarities of EP2 and EP4 Receptors

EP2 and EP4 receptor signalling have often been linked to the inflammatory response. Cigarette smoke extract increased mRNA and protein levels of EP2 and EP4 receptors in peripheral blood neutrophils from healthy volunteers, but not EP1 and EP3, at 3 hours and 24 hours by western blotting and qPCR (Profita *et al.*, 2010). Cigarette smoke extract treated neutrophils showed increased epithelial cell adhesion upon treatment with the EP2 agonist butaprost or PGE2, which was blocked by an EP4 antagonist (Profita *et al.*, 2010). Moreover, EP4 knockout mice (*PTGER4*$^{-/-}$) showed increased pulmonary inflammation in response to a range of stimuli, such as LPS and cigarette smoke (Birrell *et al.*, 2014) that were mediated by a cAMP/PKA signalling pathway. A potential explanation may be in the requirement for EP2 and EP4 receptors in the inhibition of LPS-induced cytokine release (Poloso *et al.*, 2013). In macrophages, LPS induced the expression of EP2 and EP4 receptors, potentially increasing the influence of PKA signalling in an inflammatory context (Katsuyama *et al.*, 1998; Ikegami *et al.*, 2001). However, prolonged exposure to PGE2 downregulated EP2 and EP4 expression in Chinese hamster ovary cells (Nishigaki *et al.*, 1996), suggesting a self-regulatory mechanism. Moreover, the influence of PGE2 also depends on its concentration, as a low PGE2 concentration induces pro-inflammatory IL-23 release via EP4, but higher abundance of PGE2 may block IL-23 release via EP2, which further supports the self-regulatory effect of PGE2 in resolution of inflammation through variations in receptor usage. This is further supported by the induction of Cox-2 during the resolution phase of inflammation, where the inhibition of Cox-2 may further sustain inflammation (Gilroy *et al.*, 1999; Willoughby *et al.*, 2000). Although EP2 and EP4 are highly similar in many functions and often display redundancy (Brock *et al.*, 1999), they are

structurally distinct and their expression profiles differ in response to stimuli (Arosh *et al.*, 2003; Chandramouli *et al.*, 2012; Kickler *et al.*, 2012). Moreover, EP4 only is sufficient for the PGE2-induced secretion of MMPs (Miyaura *et al.*, 2000).

1.3.2.6. FP

The FP receptor is present in the plasma membrane and the extracellular space (Belinsky *et al*., 2015). FP mRNA is present in various tissue types, amongst others the lung and whole blood. Most interestingly, the FP receptor is abundantly expressed in white blood cells (Belinsky *et al*., 2015). Activation of the FP receptor induces intracellular increases in Ca^{2+} and subsequent activation of PKC (Fortier *et al*., 2008). A potential involvement of the FP receptor subtype in inflammatory neutrophil function was demonstrated by Wallace *et al*. (2009), who showed that the FP receptor induces inflammatory gene expression of CXCL1. As this chemokine acts as a neutrophil attractant, neutrophil chemotaxis was stimulated. Moreover, LPS-induced tachycardia in mice was diminished (Takayama *et al*., 2005), while little is known about the influence of LPS/FP signalling interactions on other cellular functions in neutrophils, such as cell survival.

1.3.3. Mediation of PGE2 Survival Signalling by Nuclear Receptors.

Apoptosis in neutrophils can be engaged by different mechanisms, which all lead to the activation of an effector arm and characteristic apoptotic changes. Apoptosis can be engaged by external stimuli, such as ligand binding to the Fas receptor, or through pathogen-mediated stimuli through the TNF receptor. The intrinsic pathway can be activated by irradiation, however also passive effects, such as the withdrawal of growth factors results in apoptosis. Neutrophils are unique, as they furthermore undergo constitutive apoptosis that predetermines their lifespan, and no strategy has been found so far that would completely prevent apoptosis in neutrophils. It is known that neutrophil lifespan becomes prolonged through recruitment to sites of infection to promote pathogen clearance through release of pro-inflammatory cytokines. Also, it is well established that neutrophil survival signalling can become altered in the context of inflammation (Simon, 2003) and induced by cigarette smoke (Morozumi *et al.*, 2004). In this context, more substantial knowledge on the modulation of neutrophil lifespan in inflammation is needed to determine novel therapeutic strategies.

Nuclear receptors are a large and highly conserved family of transcription factors, which regulate gene transcription by binding to enhancers or insulators upstream of their target gene, thereby bringing remote regions of the DNA together to enable target gene transcription. Nuclear receptors possess a high structural homology, containing an AF-1 and isoform-specific domain, a DNA binding domain, a hinge domain and a ligand-binding domain. In the form of monomers, homodimers or heterodimers, nuclear receptors have well documented roles in the

regulation of cell proliferation and metabolism, the maintenance of homeostasis and in inflammatory signaling. However, the stimuli that induce their expression are highly dependent on the cell type, but require the induction of a cAMP/PKA/CREB-dependent signaling pathway (Hawk, Abel, 2011).

There are three types of nuclear receptors: Type I are classical steroid receptors that are localized in the cytosol. They are bound to inhibitory proteins in their inactive state, and will only translocate to the nucleus following ligand binding with ligands such as estrogen or glucocorticoids. In the nucleus, homodimers of Type I nuclear receptors can bind to a response element and enable target gene transcription. Type II are non-steroid receptors that can be activated by ligands such as thyroid hormones. In their inactive state, type II receptors typically already reside in the nucleus and are already bound to DNA with a corepressor. They can heterodimerize with Retinoid X receptors (RXR). Paradoxically, the identity of a ligand for type III, or orphan receptors is unknown. Orphan receptors represent half of the nuclear receptors known in humans, being founded in their discovery by cDNA library screening with degenerate primers, owing to their high structural homology. In retrospect, ligands were discovered for 70 % of the former orphan receptors (Shi, 2007) and include the endocrine receptor subfamily and several peroxisome proliferator-activated receptors, such as PPARγ. Interestingly, while all adopted orphans possess a relatively typical domain structure, the Nuclear family 4 subgroup A (NR4A) orphan receptors have bulky ligand-binding domains with a lack of binding pockets, which is thought to make those receptors incapable of classic ligand binding (Wang *et al.*, 2003; Ohkura *et al.*, 1994; Baker *et al.*, 2003; Flaig *et al.*, 2005). Nevertheless, site-specific agonists for the selective targeting of other modulatory sites, such as AF1 (Codina *et al.*, 2004; Ordentlich *et al.*, 2003) enable the current drug development for NR4A modulators, which are currently under development for NR4A2 (Dubois *et al.*, 2006; Jankovic *et al.*, 2005).

The NR4A subfamily of nuclear receptors is comprised of three members, namely NR4A1 (formerly Nurr77), NR4A2 (formerly Nurr1) and NR4A3 (formerly Nor1; Safe *et al.*, 2014). Members of this subfamily are proposed to be constitutively active and work in a ligand-independent manner by affecting gene transcription through changes in their expression levels. Signalling pathways that additively induce the expression of NR4A members are PKA and MAPK pathways (Hawk, Abel, 2011). In macrophages, but not fibroblasts, LPS-treatment induced the NF-κB-dependent expression of all three NR4A subfamily members (Pei *et al.*, 2005), suggesting that targeting NR4A family expression may a useful target in the treatment of COPD. NR4A upregulation induced by various environmental stimuli required a cAMP/PKA/CREB dependent mechanism (Kwok *et al.*, 1994; Kovalovsky *et al.*, 2002; Darragh *et al.*, 2005; Pearen *et al.*, 2006). Additionally to this, it has been shown, that nuclear receptor activity can also be altered through different posttranslational modifications via growth factor and cytokine signalling. They are primarily thought to bind to DNA as monomers.

NR4As are involved in many cellular functions including apoptosis, proliferation and metabolism. Expression of NR4As is induced by a variety of stimuli via multiple cell-signalling pathways, including PKA, NF-κB, PI3 kinase, c-jun-NH2-kinase (JNK), and MAPK pathways. The same stimuli often additionally induce further inflammatory signalling. NR4A receptors can be present in the cytoplasm and nucleus and can contribute to apoptosis in both scenarios. Their mitochondrial localisation might be linked with prostaglandin signalling as mitochondrial Cyclooxygenase (Cox2) induces inflammatory PGE2 production. Moreover, PGE2 was shown to induce NR4A gene expression via CREB and NF-κB signalling, potentially caused by a PGE2-induced CREB phosphorylation, which in turn can bind to NR4A promoters and enhance gene transcription (Holla *et al.*, 2006). The dual role of NR4A receptors on the induction and inhibition of apoptosis may be consistent with the reported dual effect of cAMP/PKA signalling on survival, and may be transmitted through the involvement of the distinct nuclear receptor family members.

1.3.3.1. NR4A1

NR4A1 is a 7946 bps gene on chromosome 15. Homozygous deletion of *NR4A1* in mice does not alter the overall mouse viability and leads to typical responses in terms of development, cell death and differentiation. A potential anti-inflammatory role in macrophagic inflammation has been proposed to be exerted through the control of IL-I β and IL-6 secretion (Bonta *et al.*, 2006). However, in macrophages, induction of NR4A1 by LPS or NF-κB increases the gene expression of pro-apoptotic and pro-inflammatory genes, i.e. caspase-4 and TNFα (Pei *et al.*, 2006). NR4A1 induces apoptosis in colon cancer cells (Wilson *et al.*, 2003). Furthermore, NR4A1 expression in T cells is also considered to be pro-apoptotic through the induction of CD30 and Fas ligand (Zhang *et al.*, 1999; Wei *et al.*, 1996), consistent with the lack of peripheral T cells in NR4A1$^{-/-}$ mice.

1.3.3.2. NR4A2

NR4A2 is a 17916 bps gene on chromosome 2. The gene product shows a predominantly nuclear localisation, but NR4A2 may be exported to the cytoplasm in response to oxidative stress. Homozygous deletion of *NR4A2* in mice induces early lethality and a failure in neuronal development. In inflammation, NR4A2 is often abnormally expressed in tissues with prolonged or inappropriate inflammation that contributes to disease pathogenesis (e.g. in synovial tissue; McEvoy et al., 2002). Moreover, inherent overexpression of NR4A2 has been associated with the development of the inflammatory phenotype in rheumatoid arthritis (Mix *et al.*, 2012; McCoy *et al.*, 2015). A large body of evidence supports a pro-survival role of NR4A2 expression, as alterations induced changes in cell survival and apoptosis in various *in vitro* and *in vivo* cell types, such as HeLa cells, neurons, in the central nervous system and NR4A2 knockdown was correlated with decreased lymphocyte counts (Ke *et al.*, 2004; Jankovic *et al.*,

2005; Li *et al.*, 2006; Saucedo-Cardenas *et al.*, 1998; Le *et al.*, 2003). A further important role for NR4A2 can be found in the regulation of neuronal differentiation, where they are overexpressed in precursor cells (Saucedo-Cardenas *et al.*, 1998; Kim *et al.*, 2002).

PGE2 and Adenosine/A2A signalling have been shown to increase the expression of *NR4A2* in colorectal cancer and rheumatoid arthritis (Holla *et al.*, 2006; Ralph *et al.*, 2005). Independent of the initial stimulus, *NR4A2* expression is universally induced through cAMP/PKA signalling and activation of CREB (Holla *et al.*, 2006; Lee *et al.*, 2004). It has been shown that the Cox2 inhibitor Rofecoxib also downregulates NR4A2, and this might be a more desirable target for non-steroidal anti-inflammatory drugs, with fewer side-effects than Cox2 inhibitors, in combination with a targeted therapy of the lungs only.

1.3.3.3. NR4A3

NR4A3 is a 41294 bps gene on chromosome 4. Homozygous deletion of *NR4A3* in mice was either lethal in embryos or induced minor behavioural defects depending on the splice variant. Nuclear NR4A3 may be linked to inflammation, as its expression is altered in the inflammation of synovial tissue (McEvoy et al., 2002) and it shows anti-inflammatory properties in macrophages through the control of IL-1β and IL-6 secretion (Bonta *et al.*, 2006). *NR4A3* knockdown reduces mast cell inflammatory gene expression of TNFα and IL-6 (Garcia-Faroldi *et al.*, 2014).

In neutrophils, NR4A3 increased superoxide generation and activated ERK (Lee *et al.* in 2000). An increase in *NR4A3* expression in the course of apoptosis has been found in several *in vitro* models, the breast cancer cell line MCF-7 (Ohkubo *et al.*, 2000), primary neurons (Ohkura *et al.*, 1994), thymocytes (Cheng *et al.*, 1997) and chondrocytes (Laflamme *et al.*, 2003). Moreover, its expression is connected to the induction of apoptosis by T cells in tumours (Woronicz *et al.*, 1994; Liu *et al.*, 1994) and the maintenance of typical cell proliferation (Mullican *et al.*, 2007). Taken together, this may suggest a pro-apoptotic role of NR4A3 in these cell types. In support, NR4A3 is expressed in smooth muscle cells (SMC) in the context of arteriosclerosis (Nomiyama *et al.*, 2006), where SMC apoptosis contributes to the formation of arteriosclerotic lesions (Mayr, Xu, 2001). The expression of *NR4A3* has found to be regulated by diverse signalling pathways, including ERK, MAPK, PI-3K, PKC, AMPK and CREB signalling (Lammi, Aarnisalo, 2008; Nomiyama *et al.*, 2006; Ohkubo *et al.*, 2000; Zhang *et al.*, 2010), with a potential involvement in differentiation and proliferation.

1.4. Aims and Objectives

Neutrophilia is a common feature in the COPD lung that contributes to disease pathology. This aspect of the disease is poorly addressed by the available treatment options, which may even further contribute to the extension of neutrophil survival in COPD.

The levels of the prostaglandin PGE2 are increased in COPD and can elicit both pro- and anti-inflammatory responses through the engagement of four different receptor subtypes. Of these, the receptor subtypes EP2 and EP4 activate the major neutrophilic pro-survival molecule PKA.

Here, it was hypothesised that the deleterious effect of PGE2 on disease pathology in COPD stem from their enhancement of neutrophil survival and our working hypothesis is that the specific inhibition of PKA survival signalling in neutrophils, will be beneficial towards the resolution of chronic inflammation in COPD.

Therefore, the specific aims of this research project are to:

1) Identify the EP receptor subtype involved in pro-survival effects of PGE2 in PMN (Chapter 3).
2) To substantiate the importance of PKA in PGE2 and LPS-mediated PMN survival in the context of COPD (Chapters 3-4).
3) To delineate the role of nuclear receptors NR4A2 and NR4A3 in PGE2-mediated PKA signalling in PMN and a murine neutrophil model (Chapters 4-5).

CHAPTER 2. METHODOLOGY

All studies on human cells were approved by the South Yorkshire Research Ethics Committee and blood was taken with informed consent of volunteers according to the regulations outlined by the regional Research Ethics Committee.

If not otherwise indicated, all protocols were undertaken at room temperature.

2.1. Cell Culture and Sample Preparation

Human peripheral blood neutrophils (PMN), murine estrogen-regulated Hoxb8 myeloid precursor cells (mCMP) and mCMP-derived neutrophils (mNØ) were cultured at 37 °C in a 5 % CO_2 humidified atmosphere.

2.1.1. Murine Estrogen-Regulated Hoxb8 Progenitor Cells (mCMP)

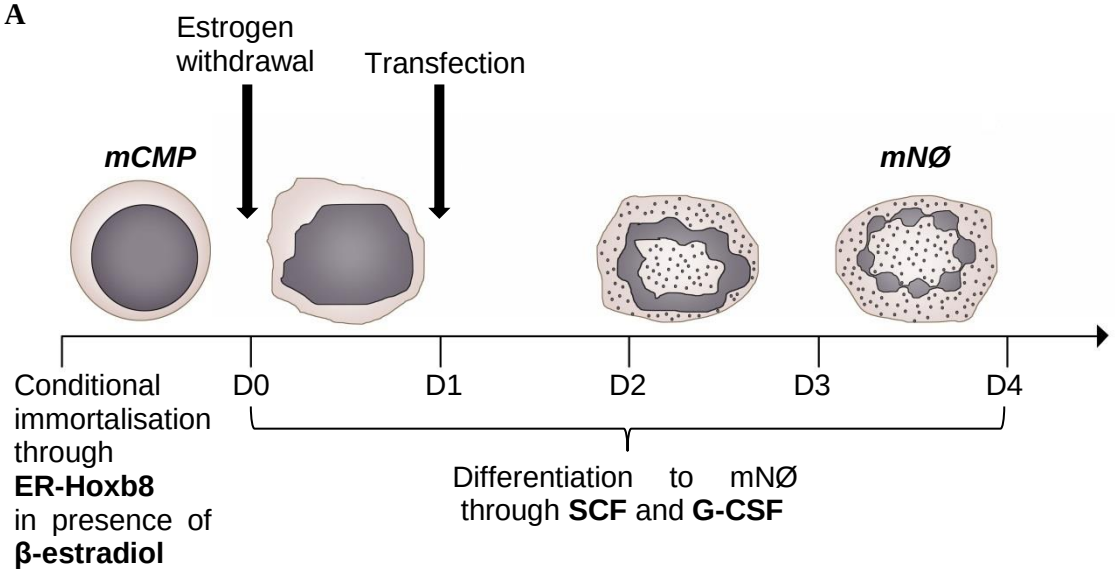
Murine estrogen-regulated Hoxb8 progenitors (mCMP) are a conditionally immortalized neutrophil precursor cell line, which yield mature murine neutrophils following a 4 day differentiation process (Wang *et al.*, 2006). Briefly, to generate mCMP, lineage-negative neutrophil precursors were obtained from the bone marrow of female BALB/c mice. The homeobox protein Hoxb8 was fused to the binding domain of an estrogen receptor and precursor cells were retrovirally transfected with estrogen-regulated Hoxb8 (ER-Hoxb8). Thus, an estrogen-regulated, chimeric variant of Hoxb8 could be expressed in mCMP, blocking the differentiation of the precursors due to the suppressive nature of Hoxb8 on myeloid cell differentiation (Knoepfler *et al.*, 2001).

mCMP (kind gift from Prof. Philip Taylor, Cardiff University) were cultured as previously described (Kirschneck *et al.*, 2011). mCMP were routinely cultured at 0.1 – 1 x 10^6 cells/ml in base medium, supplemented with 10 ng/ml recombinant murine Stem Cell Factor (SCF) and 1 µM β-estradiol to conditionally immortalise mCMP, which were typically passaged every 2 – 3 days. The base medium for mCMP and mNØ culture was OptiMEM, supplemented with 10 % HI-FCS, 1 % L-glutamine, 30 µM β-mercaptoethanol and antibiotics (1 % penicillin/streptomycin). For long term storage cells were frozen in HI-FCS with 10 % DMSO and stored in liquid nitrogen. All experiments were performed with cells between passages 2 to 8.

2.1.2. Murine mCMP derived Neutrophils (mNØ)

mCMP were differentiated into mNØ by estrogen withdrawal and supplementation with growth-factors in a 4 day differentiation protocol (**Fig. 4**; Wang *et al.*, 2006; Rosas *et al.*, 2011). Briefly, 1 x 10^6 mCMP were washed three times in 1x dPBS by centrifugation at 350g for 5 minutes to remove residual β-estradiol. Washed mCMP were resuspended in 10 ml differentiation medium, consisting of base medium (as described in chapter 2.1.1.) supplemented with 20 ng/ml of the

A



B

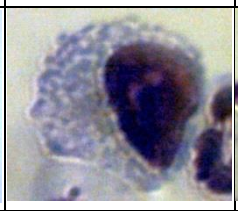
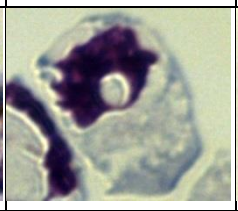
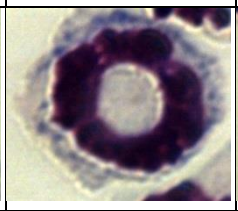
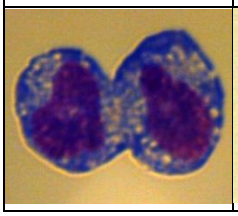
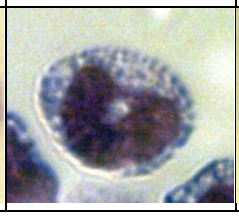
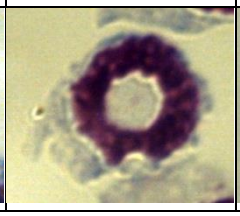
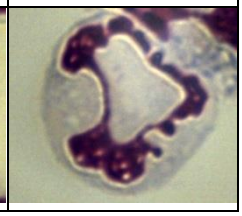
D0	D1	D2	D3	D4
mCMP	**Myelocyte**	**Metamyelocyte**	**Band mNØ**	**Lobulated mNØ**
				
				

Figure 4: ***Differentiation of mCMP to mNØ and Transfection.*** mCMP are maintained in culture in presence of SCF and β-estradiol through an estrogen regulated Hoxb8 fusion strategy, which conditionally immortalises the cells. Upon estrogen withdrawal and daily supplementation with G-CSF and SCF, mCMP differentiate into mature mNØ in a 4 day-protocol (Panel **A**). Representative images on cells on different days throughout the haematopoietic process and their respective names are displayed (Panel **B**). Transfection was undertaken at day 1 post estrogen-withdrawal. Representative images of cells in the distinct developmental stages (Panel **B**). Abbreviations: D0 – day 0, D1 – day 1, D2 – day 2, D3 – day 3, D4 – day 4, CMP – Common myeloid progenitors, mNØ – murine ER-Hoxb8 progenitor-derived neutrophils.

recombinant murine growth factors SCF and granulocyte colony-stimulating factor (G-CSF). Media and growth factors were replenished daily for 4 days, at which point >90 % of mNØ had reached maturity, as assessed on the basis of mNØ morphology by light microscopy.

For all subsequent assays, mNØs were cultured in 96-well Flexiwell plates at 0.5 x 10^6 cells/well with or without treatment of SCF (20 ng/ml), G-CSF (20 ng/ml), Q-VD-OPh (QVD; 0.1 μM), dibutyryl cAMP (dbcAMP; 100 μM), staurosporine (STS; 500 μM), N6-MB-cAMP (100 μM) and 8-AHA-cAMP (100 μM). Appendix 1 contains a comprehensive list of all reagents used in this study. All experiments were performed in triplicate and each n originated from mNØ generated from independent passages of mCMP. mNØ used in this study were derived from mCMP cultures that did not exceed passage 8.

2.1.3. Human Peripheral Blood Polymorphonuclear Neutrophils (PMN)

2.1.3.1. Isolation through Percoll Density Gradient Centrifugation

PMN were isolated from peripheral blood of healthy volunteers (**Table 2**) through dextran sedimentation and Percoll density gradient centrifugation, as previously described (Sabroe *et al.*, 2003; Vaughan *et al.*, 2007). Briefly, to avoid coagulation, 40 ml of freshly sampled blood from human subjects was collected in citrated tubes (5 ml 3.8 % tri-sodium citrate [w/v]), obtaining a final concentration of 0.48 % tri-sodium citrate (w/v). Great care was taken during the procedure to avoid all unnecessary turbulence in the liquid, to reduce cell activation. Moreover, saline and HBSS buffer used in the procedure were Ca^{2+}/Mg^{2+} free to prevent cell activation.

Citrated blood was centrifuged at 270 g for 20 minutes to separate the cellular component from plasma, yielding an upper and lower phase. The upper phase, containing platelet rich plasma, was removed and centrifuged at 1155 g for 20 minutes to obtain platelet poor plasma (PPP). To the lower phase, 6 ml of a 6 % (w/v) dextran/saline (Ca^{2+}/Mg^{2+} free) solution was added to obtain a final concentration of 0.5 % (w/v) dextran. Bubbles were carefully removed with a Pasteur pipette to prevent neutrophil activation and the lid replaced loosely to avoid subsequent disruption of the interface. Thereafter, red blood cells were allowed to sediment for 20 - 30 minutes, until a clear interphase had formed. The supernatant was then carefully taken off and centrifuged at 185 g for 6 minutes to pellet the white cells.

The cell pellet was resuspended in 1.5 ml PPP and layered onto a discontinuous plasma-Percoll density gradient, which was composed of two layers, as follows: The upper layer contained 42 % Percoll and 58 % PPP and was carefully layered onto the lower layer, which contained 51 % Percoll and 49 % PPP. The Percoll gradient was centrifuged at 225 g for 11 minutes (brake at 0). Based on their distinct densities, red blood cells accumulated at the bottom of the tube, PMN on top of the 51 % Percoll/PPP layer, and peripheral blood mononuclear cells (PBMC) on top of the 42 % Percoll/PPP layer (**Fig. 5**). PBMC and PMN populations were

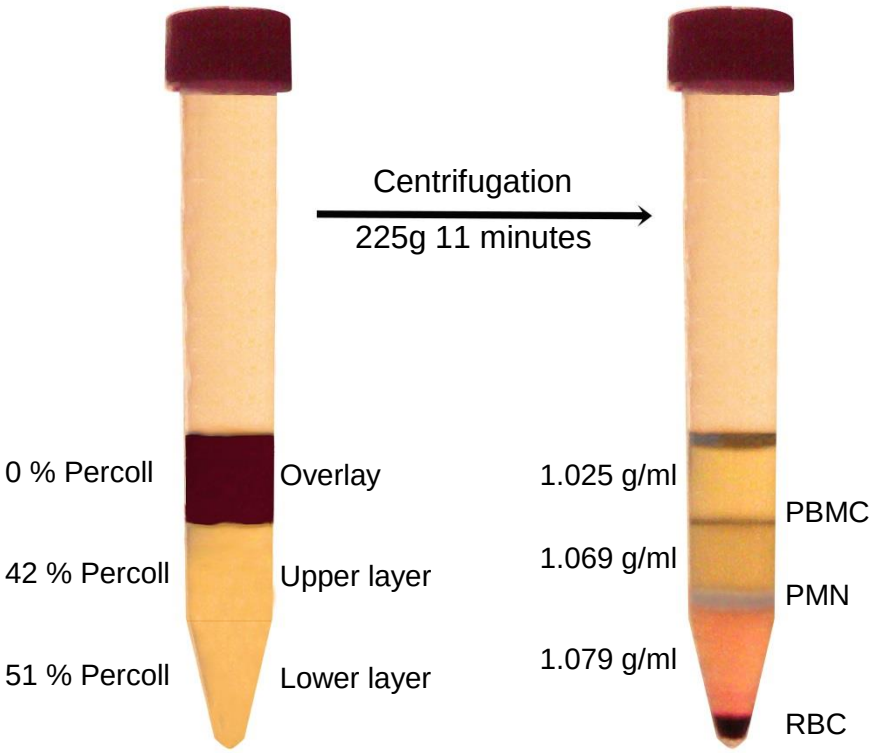


Figure 5: ***Percoll Density Gradient Centrifugation and Abundance of Blood Cells in Whole Blood.*** A biphasic Percoll density with a lower layer containing 51 % Percoll and an upper layer containing 42 % Percoll was overlayed with 1.5 ml of a cell pellet/PPP suspension. Following the centrifugation at 225g for 11 minutes, cells accumulated in the layers according to their respective densities (as outlined in **Table 2**).

Table 2: Presence and density of blood cell types in human blood.

Cell types	White blood cells (%) present in whole blood	Density (g/ml)
Monocytes	3 %	1.05 - 1.07
Neutrophils	70 %	1.08 – 1.10
Lymphocytes	25 %	1.06 - 1.08
Basophils	1 %	1.07 - 1.08
Eosinophils	1 %	1.09 – 1.10
Erythrocytes	N.A.	1.11 – 1.12

carefully extracted, resuspended in 5 ml PPP and diluted with HBSS buffer (Ca^{2+}/Mg^{2+} free) to a final volume of 40 ml. A haemocytometer count was performed and subsequently, the cell suspension was centrifuged at 420 g for 6 minutes. The cell pellet was resuspended at a density of 5 x 10^6 cells/ml in standard culture medium.

2.1.3.2. Negative Magnetic Selection of Percoll Purified PMN.

In experiments, where highly pure PMN were required, neutrophil purity was increased by negative magnetic selection using a custom antibody cocktail. As described in Sabroe *et al.* (2003), negative magnetic selection with antibodies against CD2, CD3, CD9, CD19, CD36, CD56 and glycophorin A was used to increase the purity of the Percoll pure PMN preparation to yield neutrophils of a purity > 97%. For the remainder of this thesis negatively selected neutrophil populations will be referred to as highly purified PMN. In all experiments, highly purified PMN were cultured at 1.67 × 10^6/ml in RPMI, supplemented with 10 % HI-FCS, 1 % L-glutamine and antibiotics (1 % penicillin/streptomycin; Invitrogen) in 96-well Flexiwell plates.

2.1.3.3. Cell Culture

For all assays, freshly isolated PMN were cultured in 96-well Flexiwell plates at 0.25 x 10^6 cells/well and stimulated with different pro- and anti-apoptotic factors as indicated in the figure legends. Appendix 1 contains a comprehensive list of all reagents used in this study. The start of the incubation was designated as 0 hours.

Where appropriate, antagonists were preincubated for 15 minutes before addition of agonists. All experiments (apart from the optimisation of mNØ nucleofection) were performed at least in triplicate with cell preparations obtained from independent donors.

2.1.3.4. Supernatant Co-Culture Assays

In PMN supernatant co-culture experiments, 100 µl stimulated media was incubated for 1 hour with or without 50 µl highly pure PMN (0.5 x 10^6 cells/ml plain media) at 37 °C in a humidified atmosphere. Cell-free supernatants were collected after centrifugation of the samples at 350 rcf for 2 minutes. Thereafter, 100 µl PMN (0.5 x 10^6 cells/ml plain media) from the same donor were incubated with 50 µl of collected media or PMN supernatants (1:3 dilution) and incubated for a further 3 hours, before neutrophil apoptosis was quantified.

2.2. Quantification of Neutrophil Apoptosis

Neutrophil apoptosis was assessed morphologically by modified Wright-Giemsa Staining. Additionally, flow cytometry was used to determine phosphatidylserine (PS) exposure by PE-annexin V staining and membrane integrity by ToPro-3 staining.

2.2.1. Light Microscopy

Apoptosis was first defined on the basis of morphological features, with one of the most prominent being chromatin condensation and cell shrinkage (Kerr *et al.*, 1972). Although it has been shown that the distinct cellular changes connected with apoptosis are in fact caused by independent mechanistic processes (Lu *et al.*, 2005), it remains well accepted that the occurrence of several stereotypical morphological changes of the cells constitutes apoptotic cell death. Therefore, light microscopy is recommended as a standard procedure in apoptosis research, since apoptotic features can be accurately visualised and aid in the detection of neutrophil apoptosis. Chromatin condensation and cleavage, along with other morphological changes associated with apoptosis, such as cell shrinkage and cell blebbing were visualised and quantified by microscopic inspection of cytospins, stained by the Romanowsky method (Romanowsky, 1891).

Briefly, cells were spun onto microscope slides assembled onto cytocentrifuge chambers in a Cytospin centrifuge (Shandon Inc.) for 3 minutes at 300 rpm. Subsequently, cells were fixed with 100 % methanol, until the alcohol had evaporated. Slides were then immerged in Pink Diff-Quik dye for 1 minute to stain cytoplasmic organelles, and then transferred to Blue Diff-Quik dye for 3-5 mins for nuclear staining. Following the removal of excessive dye by a wash in water, slides were air dried and coverslips were mounted onto fully dry slides with DPX mounting medium. Apoptosis was assessed by microscopy with a 100x oil-immersion magnifying lens, where 300 cells were typically counted per slide. Cell purity was assessed by assessing 500 cells on each of two slides. Absolute cell numbers were counted by haemocytometer counts and tryphan blue exclusion. All experiments performed in this dissertation were performed with cells of purity > 90% or 97% for highly pure PMN.

2.2.2. Flow Cytometry

Early apoptotic cells expose phosphatidylserine (PS) on the outer leaflet of the plasma membrane, which is a critical event preparing them for removal by tissue macrophages (Fadok *et al.*, 2001; Hampton *et al.*, 2002). For this purpose, the PS probe Annexin V conjugated to the fluorochrome phycoerythrin (PE) was used to bind to the epitope PS on apoptotic neutrophil cell surfaces. Simultaneous staining with the viability dye ToPro-3, detecting cytolysis, is therefore used to discriminate between healthy, apoptotic and necrotic cells (**Fig. 6**). The addition of CountBright beads allows the estimation of the total number of viable cells present in the sample.

Briefly, for PE-annexin V/ToPro-3 staining, cells were collected from cell culture plates and washed in 1x DPBS at 350 rcf for 2 minutes in a tabletop microcentrifuge. Thereafter, cells were resuspended in 50 µl 1x Annexin binding buffer. Upon addition of 2.5 µl PE-annexin V (1:40), samples were incubated for 20 minutes in the dark. Thereafter, 50 µl of a 1:5000 dilution of

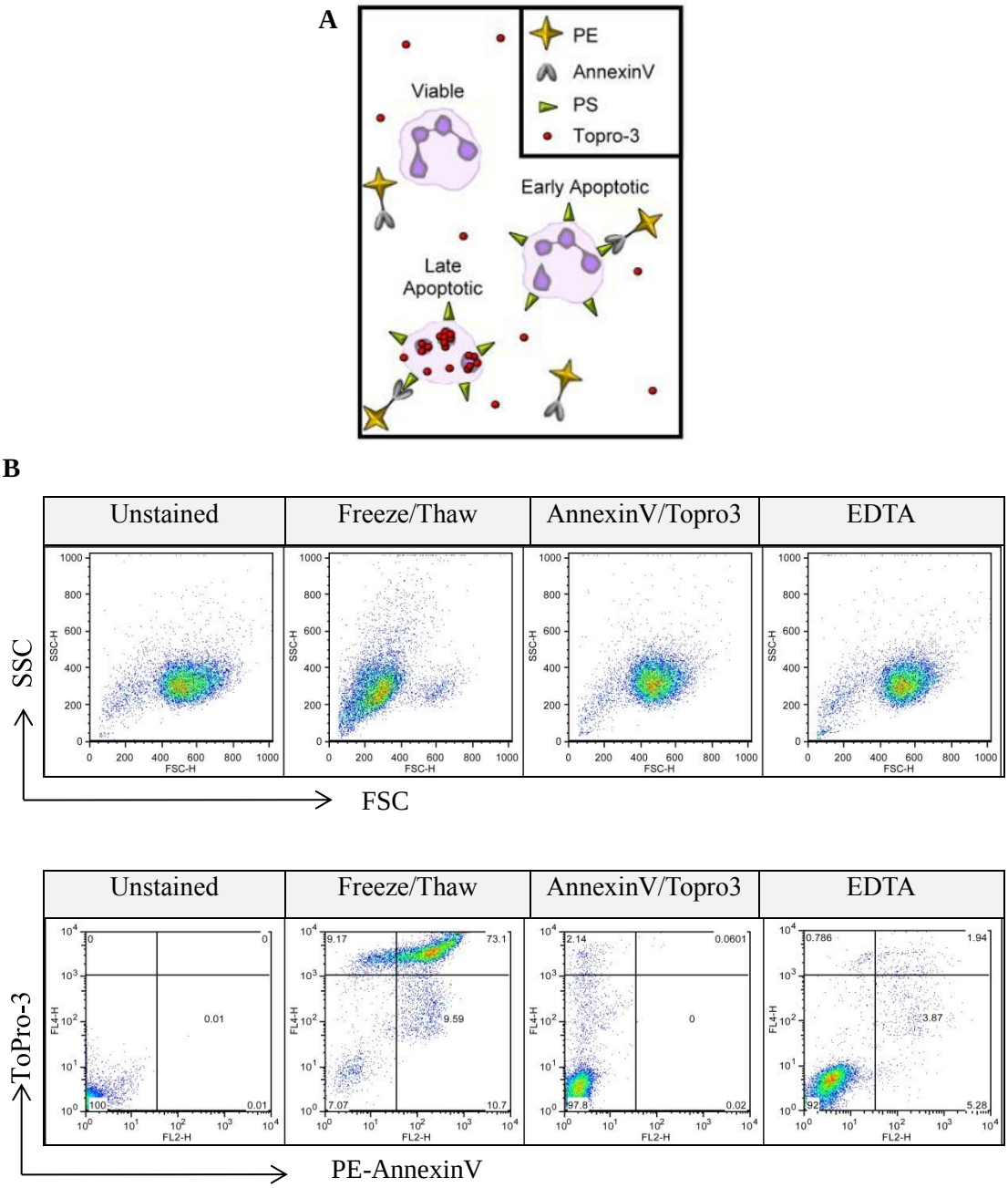


Figure 6: ***Flow Cytometric Measurement of Cell Death in mNØ.*** Viable cells possess an intact cellular membrane, and do not expose PS on their cell surface. (Panel **A**). In early apoptotic stages, cells still maintain membrane stability, but expose PS, to which PE-annexin V binds. With increasing length of culture of apoptotic neutrophils, the cell membrane gradually starts degrading and allows the leakage of the membrane DNA intercalating dye ToPro-3 into the cell. Representative flow cytometric images of Gating controls for annexin V/ToPro-3 stained neutrophils (Panels **B**, **C**). Unstained controls show a high percentage of viable cells in a tight population of SSC/FSC scatter plots (Panel **B**) with a low level of autofluorescence for FL2/FL4 (Panel **C**). Necrotic cell death was modelled by freezing the samples at – 80 °C for 2 minutes prior to the addition of antibodies, which induced the formation of substantial amounts of cells with decreased size and cellular debris on SSC/FSC scatter plots, while most cells were highly fluorescent for ToPro-3. EDTA controls were aimed to determine the levels of unspecific binding of PE-annexin V, as EDTA is a calcium chelator, which uses calcium in competition for the calcium dependence of the annexin V/PS binding.

ToPro-3 (total 1:10000) in annexin binding buffer, as well as 10 µl of well vortexed CountBright counting beads were added, before the sample was analysed on FL-2 and FL-4 (log-scale) with a FACSCalibur flow cytometer and CellQuest Software. The following served as controls for flow cytometry:

- An unstained control for background FL-2 fluorescence.
- A calcium chelated control through addition of 2 µl 0.5 M EDTA to inhibit the calcium-dependent binding of Annexin V to phosphatidyl serine.
- A freeze/thaw control, which was incubated for 2 minutes at -80°C before addition of fluorescent dyes, to create cell populations with permeabilised membranes.

2.3. mRNA Transcript Modulation and Quantification

2.3.1. Trizol RNA Extraction

Total RNA was extracted from cells using TRI reagent. TRI reagent is a commercially available adaptation of the guanidinium isothiocyanate-phenol-chloroform extraction method (Chomczynski, 1989). According to this method, cells are initially lysed in TRI reagent, which acts as a denaturing solution. In this way, the protein denaturant guanidine thiocyanate denatures ribonucleases and eliminates remaining RNase activity. Centrifugation of the sample fractions it into a lower organic phase and an upper aqueous phase. In contrast to DNA and proteins, RNA remains water soluble in the acidic conditions in the sample, and can therefore be isolated and precipitated from the aqueous fraction.

Briefly, cells in 150 µl culture media were homogenised in a 1:3 ratio of TRI reagent/cells. Addition of 5 % 1-bromo-3 chloropropane, incubation for 2 - 5 minutes and centrifugation at 12,000 rcf for 15 minutes at 4 °C separated protein into a lower organic phase, DNA in an interphase and RNA in the top aqueous phase. RNA in the aqueous phase was precipitated with 0.5 ml 100% isopropanol, incubated for 5-10 minutes and centrifuged at 12,000 rcf for 10 minutes. Ultimately, RNA was washed twice in 75% ethanol. The resulting RNA was treated with a DNase free Kit as per manufacturer's instructions, to eliminate contaminating DNA from the sample. To this end, 2 Units rDNase I were added to the DNA and the sample was incubated for 20-30 minutes at 37 °C.

Following this, rDNase I was inactivated by DNase Inactivation Reagent and incubated for 2 minutes. Sample concentration was determined by Nanodrop. The expected value for A260/A280 was < 1.9-2.0.

2.3.2. Reverse Transcription PCR

1 µg of DNAse-treated, total RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions. Briefly, random amplification primers were used to initiate the first-strand cDNA synthesis in a buffer

containing 5 mM dNTP, 100 U Multiscribe Reverse Transcriptase and 1 µl RNAse Inhibitor in a total volume of 40 µl. Samples were then incubated for 10 minutes at 25 °C, 2 hours at 37 °C, and 5 minutes at 85 °C for the reverse transcription reaction to occur.

2.3.3. Conventional PCR

1 µl of the synthesised cDNA reaction (as described in chapter 2.3.2.) was used for conventional PCR. Conventional PCR was conducted in GoTAQ Green Flexi Buffer in a final volume of 25 µl, using GoTAQ Flexi DNA Polymerase with 1 mM $MgCl_2$, 12.5 µg BSA and 200 nM of each PCR primer. Primers were designed to include at least one exon-exon junction spanning using PrimerBlast (NCBI). Primer sequences can be found in appendix 5. The PCR cycling conditions for the amplification of the desired products were initiated by a 2 minute denaturation at 94 °C. PCR reaction was performed using 30-37 cycles of a denaturation of 94 °C for 30 seconds, 1 minute annealing at the primer specific annealing temperature (see appendix 5), and 30 seconds of elongation at 72 °C. This was followed by a final elongation of 2 minutes at 72 °C. PCR products were visualized on a 1.2 % TAE agarose gel, stained with ethidium bromide. PCR products were quantified by densitometry using ImageJ software and normalised to the expression of endogenous cyclophilin B (*CypB*) or Glyceraldehyde-3-Phosphate (*GAPDH)*, as well as an internal calibrator, such as the media or 0 hours control, as indicated.

2.3.4. Quantitative RT-PCR (qPCR)

Taqman qPCR is a PCR based amplification strategy, by which changes in gene expression (mRNA) can be analysed, and PCR product amplification tracked in real time. To this end, cDNA derived from a reverse transcribed mRNA (see chapter 2.3.2) is amplified by using a probe for the target sequence, tagged with the fluorophore 6-carboxylfluorescein (FAM) and the quencher tetramethylrhodamine (TAMRA). Product amplification degrades the primer and thus releases FAM, which is no longer quenched by TAMRA. Therefore, released FAM can be detected through Fluorescence Resonance Energy Transfer (FRET) in the qPCR cycler, and is directly proportional to the amount of cDNA present in the sample.

qPCR was performed with 1 µl cDNA template on the ABI PRISM 7900HT Sequence Detection System (Life Technologies). In a reaction volume of 20 µl, 1x qPCR MasterMix Plus with hotstart enzyme HotGoldStar, 1x Taqman gene expression array (Applied Biosystems), sequence specific primer probes (FAM/TAMRA), 1 µl cDNA template and water were mixed. The qPCR reaction was recorded in real-time using an ABI 7900 automated TaqMan System (Applied Biosystems) using the following cycles: 50 °C 2 minutes, 95 °C 10.25 minutes, 60 °C 1 minute for 40 cycle repeats. Samples were run in duplicates and were quantified as indicated using the $2^{-\Delta\Delta CT}$ method, by Taqman standard curves against known plasmid copy numbers or

against an arbitrary standard (PBMC cDNA pooled from 5 samples) through the SDS software (Applied Biosystems). Data were normalised to expression of endogenous *GAPDH*. For $2^{-\Delta\Delta CT}$, statistical analysis was performed on values normalised against the endogenous control (ΔCT), and displayed on the corresponding graphs for $2^{-\Delta\Delta CT}$. Hereby, ΔCT was the difference between CT(target gene) and CT(GAPDH), as defined by the formula:

$$\Delta CT = CT(\text{target gene}) - CT(\text{reference gene; GAPDH})$$

The ΔCT of all tested conditions were normalised to the internal control, or calibrator by using:

$$\Delta\Delta CT = \Delta CT(\text{test}) - \Delta CT(\text{calibrator; media/0 hours})$$

The relative change in gene expression can be expressed as

$$Ratio: \left(\frac{\Delta CT(test)}{\Delta CT\ (calibrator)}\right) = E^{\Delta CT(calibrator) - \Delta CT\ (test)}$$

With the efficiency of the reaction being defined as $E = 10^{\frac{-1}{slope}}$, and a perfect slope with $slope\ (perfect) = -\pi$, it follows that $E = 10^{\frac{-1}{-\pi}} = 2$. Thus:

$$Ratio: \left(\frac{\Delta CT(test)}{\Delta CT\ (calibrator)}\right) = 2^{\Delta CT(calibrator) - \Delta CT\ (test)} = 2^{-\ \Delta\Delta CT(test/calibrator)}$$

2.3.5. Primer Sequences

All oligonucleotides for conventional PCR used in this study (Appendix 5) were synthesised in solid-phase with a Silyl-phosphoramidite method by Sigma. Primer design was by PrimerBlast (NCBI), aiming at a GC content of ~60%, introduction of a GC clamp and avoiding secondary structures and repeats. Primers were designed to include at least one exon-exon junction spanning. For all qPCR reactions, the primer/probe sets in **Table 3** were used.

2.3.6. Nucleofection and RNAi mediated mRNA Knockdown

The RNA interference pathway is an epigenetic modulation strategy, allowing the transient silencing of gene expression levels. The RNAse Dicer converts long double-stranded RNAs (dsRNA) into short double-stranded interference RNAs (siRNA). siRNA is catalytically unwound and the thus separated antisense strand is integrated into the RNA-induced silencing complex (RISC). Homologous mRNA is recognised and degraded by RISC-activated ribonucleases, depleting overall mRNA levels for the specific gene product.

siRNA was introduced into the cell by Nucleofection, which is an electroporation strategy. For this, the many hydrophobic pore openings in the hydrophobic bilayer cell membrane are converted into hydrophilic pores through an electric pulse, leading to rearrangements of the lipids in the pore composition, so that highly charged RNA is enabled to pass through. Nucleofection (**Fig. 7**) was carried out according to the manufacturer's protocol with lyophilised

Table 3: qPCR primer/probe sets by Applied Biosystems used in the study.

qPCR Primer Probe Set	RefSeq (Translated Protein)	Exon Boundary	Assay Location	Amplicon Length
GAPDH	NM_001256799.1 (GAPDH isoform 2)	6-7	728	93
	NM_002046.4 (GAPDH isoform 1)	7-8	704	93
NR4A2	NM_006186.3 (NR4A2)	5-6	1578	69
NR4A3	NM_006981.3 (NR4A3 isoform a)	4-5	1816	68
	NM_173200.2 (NR4A3 isoform b)	5-6	1888	68
	NM_173199.2 (NR4A3 isoform c)	4-5	1816	68
PTGER2 (EP2)	NM_000956.3 (EP2)	1-2	1089	92
PTGER4 (EP4)	NM_000958.2 (EP4)	2-3	1457	68

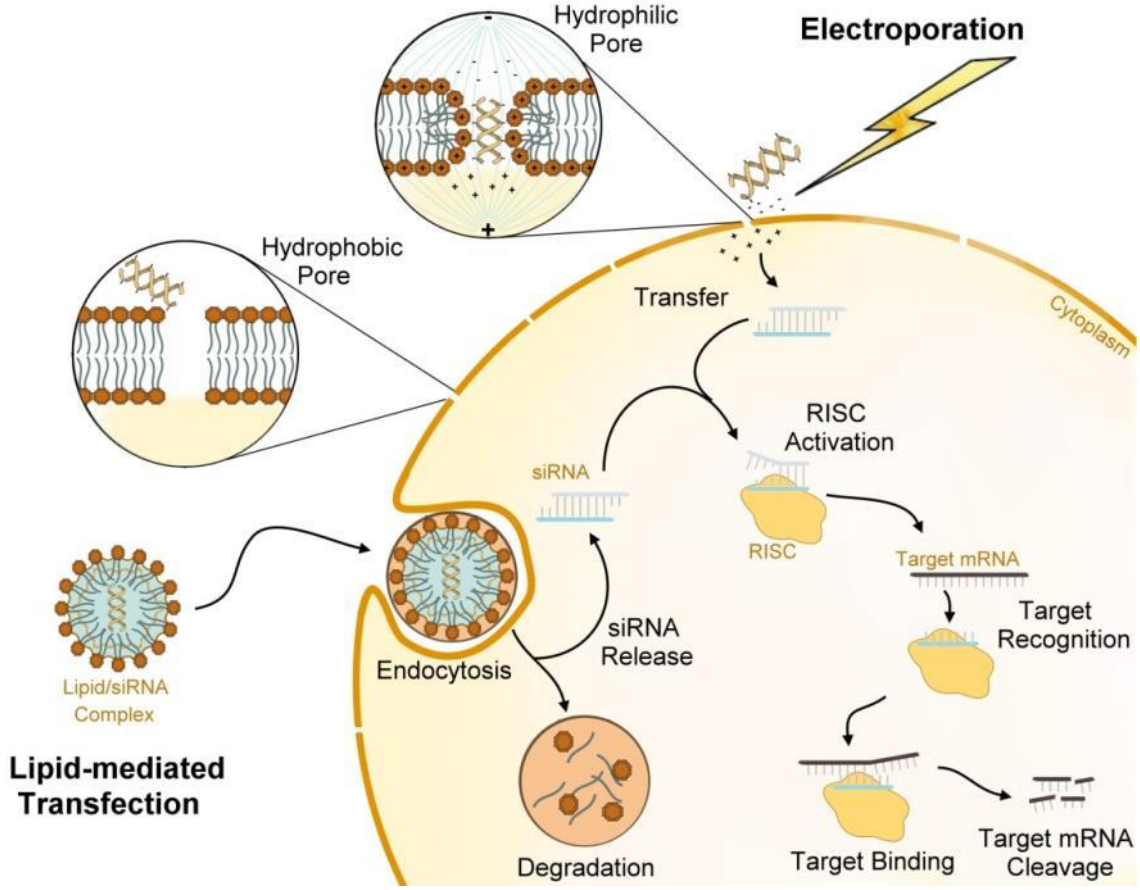


Figure 7: ***Mechanism of siRNA-mediated Gene Silencing.*** Electroporation or Lipid-based reagents can be employed to mediate the transfer of the coding sequence for specific siRNA into cells. While electroporation changes the composition of pores, lipids enclose the siRNA and are transferred into the cell by endocytosis, and the lipids degraded in the lysosome. The siRNA reaches the nucleus and integrates into host DNA sequences. Transcription and export of the resulting siRNA into the cytoplasm enables the formation of a siRNA/RISC complexes, resulting in the binding and recognition of the single stranded siRNA to its target sequence, ultimately resulting in depletion of siRNA products for the gene of interest.

siRNA (20 μM), with the Amaxa Nucleofector Kit V.

Briefly, $1x10^6$ cells were washed 3x in PBS and resuspended for 2 minutes in 100 μl of supplemented Nucleofector Solution and 3 μg of a target-specific siRNA pool or a scrambled control. Suspensions were transferred into cuvettes, nucleofected with program D-023 and immediately recovered in 1 ml of fresh growth medium. For mCMPs, cells were then maintained in 10 ml media for 48 hours until their harvest. For mNØs, cells were transferred into 10 ml differentiation media and media was replenished daily until they were harvested following differentiation for 3 or 4 days. Knockdown efficiency was assessed by qPCR.

2.4. Immunostaining

2.4.1. Immunocytochemistry (ICC) & Confocal Microscopy

PMN cytospins were prepared (as per section 2.3.1) and fixed using 100 % MeOH. A wax pen (Sigma) was used to define a (hydrophobic) boundary around the cells. Slides were blocked for 1 hour using 20 % goat serum, and subsequently incubated with polyclonal PE-conjugated antibodies against EP2 (4 μg/ml, IMG-71915, Cayman Chemicals), EP4 (5 μg/ml, 115-01, Cayman Chemicals) or IgG (5 μg/ml, Columbia Biosciences) for 1 hour at 4 °C in a humidified chamber. Cytospins were rinsed 3 times for 5 minutes in PBS before incubation for 1 hour at 4 °C with 5 μg/ml DAPI. Subsequently, coverslips were mounted with Fluoromount mounting medium (Sigma) and stored at 4 °C in aluminium foil. Light exposure was avoided during incubation and storage of the stained cytospins to circumvent photobleaching. Slides were examined in triplicate for each condition by confocal microscopy on a Nikon A1 Confocal Microscope with the LIS Elements software.

2.4.2. Western Blotting

Western blotting is a well-established technique, used to separate proteins according to their size on SDS-PAGE gels, and fix proteins on membranes. For the analysis of protein expression in highly pure PMN, whole cell lysates were extracted, separated on 10 % SDS-PAGE gels and transferred to nitrocellulose membranes using semi-dry transfer.

2.4.2.1. Whole Cell Lysate Preparation

PMN were isolated through Percoll density gradient centrifugation, as described in chapter 2.1.3. Total cell numbers were determined by haemocytometer counts. Neutrophils contain a high amount of diverse proteases in their granules (Pham, 2006). Therefore, the serine protease inhibitors phenylmethanesulfonylfluoride (PMSF) and diisopropyl fluorophosphate (DFP), which preferentially inhibit trypsin and esterases, respectively, were employed in addition to a cocktail of diverse protease inhibitors (PI) during the cell lysis procedure.

For every sample, 5 x 10^6 PMN were pelleted by centrifugation at 350 g for 2 minutes and then washed once in PBS. Cells were lysed in 50 µl of cold lysis buffer, consisting of 0.5 µl DFP, 0.5 µl PMSF, 0.5 µl PI and 48.5 µl water. Cells were incubated on ice for 1 minute to allow complete lysis to occur. After the addition of 50 µl 2x SDS loading dye, samples were boiled for 10 minutes to degrade secondary structures, such as disulphide and hydrogen bonds, which might introduce alterations in their polarity or structure. Lysates were stored at – 20 ºC until required. All buffer recipes can be found in appendix 2.

2.4.2.2. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a widely used method for the size-dependent separation of proteins. A 10 % SDS-PAGE gel was cast by pouring a stacking gel onto a polymerised resolving gel (**Table 4**) in a gel casting frame, assembled according to the manufacturer's instructions. Briefly, glass plates were cleaned with alcohol before use, and assembled in the gel casting frame. Reagents in black (**Table 4**) for the resolving and stacking gels were mixed in consecutive order, as indicated in **Table 4**. Addition of APS and TEMED was delayed until the respective gels were poured to prevent premature polymerization. Firstly, the resolving gel was poured into the casting frame, overlayed with isopropanol and allowed to polymerize. Isopropanol was poured off, and the plates rinsed with water. Thereafter, APS and TEMED were mixed with the prepared stacking gel reagents, mixed well, and pipetted onto the polymerised resolving gel. A comb was added, and the gel was allowed to solidify. Thereafter, combs were removed and the gel was placed into a gel tank with 1x running buffer (Appendix 1). After the wells were rinsed with running buffer, 20 µl of PMN lysate (~1 x 10^6 PMN) was loaded into individual lanes of the 10% SDS-PAGE gel. An electric current of 100 V was introduced to the gel tank to stack proteins above the resolving gel layer, after which the current was increased to 150 V. The uniform charge imposed onto the proteins thereby allows for their size-dependent separation in the resolving gel. A molecular weight marker was used to estimate the respective band sizes.

Table 4: Recipe for 10% SDS-PAGE gel, to obtain resolution of 30-200 kDa proteins.

Reagent	**10% Separating Gel (4 gels)**	**4% Stacking Gel (4 Gels)**
H_20	6.3 ml	3.05 ml
1.5M Tric-HCl, pH 8.8	4 ml	
0.5M Tric-HCl, pH 6.8		1.25 ml
30% Acrylamide	5.4 ml	0.67 ml
10% SDS	160 µl	50 µl
10% APS	160 µl	50 µl
TEMED	16 µl	12 µl

2.4.2.3. Semi-Dry Protein Transfer

Proteins on SDS-PAGE gels were transferred to nitrocellulose membranes through semi-dry transfer with transfer buffer (as described in appendix 2) on a G2 Fast Blotter (Pierce). Briefly, an SDS-PAGE gel was removed from between the glass plates and placed into transfer buffer. Similarly, pre-cut blotting paper (Whatman) was likewise soaked in transfer buffer. The layers for semi-dry blotting were assembled on the blotter as follows: a piece of pre-wet nitrocellulose membrane was placed onto 3 pieces of soaked Whatman paper. The SDS-PAGE gel was carefully positioned onto the nitrocellulose membrane and covered by three pieces of soaked Whatman paper. Transfer buffer was used to moisten the stack, before air bubbles and excess liquid were removed by rolling the layers lightly. The G2 Fast Blotter was assembled, as per manufacturer's instructions and proteins were transferred for 7 minutes at 25 V, for 1 single gel.

2.4.3. Immunostaining of Western Blots

Protein expression for the target proteins was determined through immunostaining with target-specific antibodies, chemiluminescent exposure of the stained western blots and densitometric analysis.

2.4.3.1. Immunostaining

Membranes were blocked for 1 hour in 5 % milk/TBS-0.1 % Tween-20 at room temperature. Primary antibodies for EP2 (1:200) and EP4 (1:100) in 5 % milk/TBS-0.1% Tween-20 were incubated with the blots at 4 ºC overnight. Before incubation with appropriate HRP conjugated secondary antibodies in 5 % milk/TBS-0.1 % Tween-20 for 1 hour, blots were washed 4 times in TBS/0.1% Tween-20 for 10 minutes. Bands respective to the target (EP2 – 53 kDa; EP4 – 65 kDa/52 kDa) were visualised by chemiluminescence through the Clarity Western ECL Substrate Kit using the Chemidoc system. Western blots were incubated with Ponceau S solution and washed in TBS/0.1% Tween-20, before western blots were re-probed with antibodies for β-actin (1:10,000), as a loading control. Densitometry of the exposed blots was performed using the ImageJ software.

2.4.3.2. Stripping and Reprobing

To strip the membranes, 0.2 M NaOH was incubated on the membranes for 15 minutes, before it was diluted with 3 washes in dH_20 and TBS/0.1 % Tween-20, before the membrane was blocked and reprobed.

2.5. Statistical Analysis

Data were analysed using the Prism 6.04 software (GraphPad). Statistical tests were performed as appropriate and included the following: Two-way ANOVA, One-way ANOVA, Student's t-test and a post hoc test, as indicated. Comparisons between data sets with one or two variables were performed through One-Way or Two-Way ANOVA respectively. Student's t test was used to compare the differences of two independently treated data points with a single variable. Bars show mean ± SEM. Differences to controls are indicated by asterisks (*), and differences between treatments are indicated by octothorpes (#). For cytospins 300 cells were typically counted per timepoint, while for flow cytometry 10,000 events were accumulated per sample. Statistically significant comparisons were illustrated as follows: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

CHAPTER 3. MECHANISMS OF PGE2 SURVIVAL IN LPS PRIMED PMN.

LPS and PGE2 are both potent mediators of neutrophil survival in the context of COPD (Dick *et al.*, 2009; Ottonello *et al.*, 1998; Profita *et al.*, 2010). Inhalation of LPS has been shown to mimic inflammatory characteristics of COPD and disease exacerbations (Korsgren *et al.*, 2012; Kobayashi *et al.*, 2013), potentially through the induction of inflammatory gene expression by TLR signalling (Sabroe *et al.*, 2003). LPS and PGE2 can play a cooperative role *in vivo.* LPS induces monocyte PGE2 expression via Cyclooxygenase 2 (Cox2; Amann *et al.*, 2001; Ikegami *et al.*, 2001; Hofer *et al.*, 2004). This increases the amount of free intracellular PGE2, and monocytes might thereby serve as a major source of prostaglandin in the lung (Fogel-Petrovic et al., 2004). PGE2 is also known to be highly increased in the induced sputum of smokers, corresponding with an elevation in Cox2 expression in COPD patients and smokers (Profita *et al.*, 2010). However, there is contradictory evidence towards the impact of PGE2 on airway inflammation (as discussed in chapter 1.3.). Briefly, both pro- and anti-inflammatory functions have been reported for various prostaglandins, such as PGD2, its metabolites (Sandig *et al.*, 2007) and PGE2 (Akaogi *et al.*, 2006; Frolov *et al.*, 2013), potentially through the engagement of the EP4 receptor subtype (Birrell *et al.*, 2015). Divergently, pro-inflammatory effects of PGE2 in various inflammatory conditions have likewise been reported (Sheibanie *et al.*, 2007) and proposed (Ward *et al.*, 2004). Furthermore, PGE2 induced PMN survival is implicated in the occurrence of neutrophilia, which can exacerbate inflammatory conditions.

Stimulation of blood neutrophils with LPS transiently upregulated their TLR2 and TLR4 receptor expression (Baines *et al.*, 2011). Additionally, the Sabroe/Whyte research groups have previously shown that LPS-stimulation of neutrophils only transiently increased their survival via TLR4 and NF-κB activation with an observed loss of TLR4 responsiveness after the immediate immune responses (Sabroe *et al.*, 2002a). Thus, TLR4 desensitization would be expected to abrogate LPS induced neutrophil survival. Surprisingly, neutrophil survival at a late timepoint was sustained following TLR4 desensitization, where LPS required the presence of monocytes to elicit a biological response (Sabroe *et al.*, 2002a). This was attributed by the authors to indirect effects on PMN survival through secretion of survival factors by monocytes.

Here, the effects and mechanisms of PGE2 in PMN survival were examined. It was hypothesized that LPS increases PKA-dependent survival responses in PMN through the modulation of prostaglandin receptor expression. This might increase the susceptibility of PMN to LPS-induced PGE2 secretion by monocytes. The first aim of this chapter was to determine the prostaglandin receptor responsible for mediating survival effects of PGE2 and secondly, to understand the role of LPS in priming PGE2 induced survival.

CHAPTER 3

3.1. Identifying the Receptor Subtypes Involved in the Pro-Survival Effects of PGE2 in PMN.

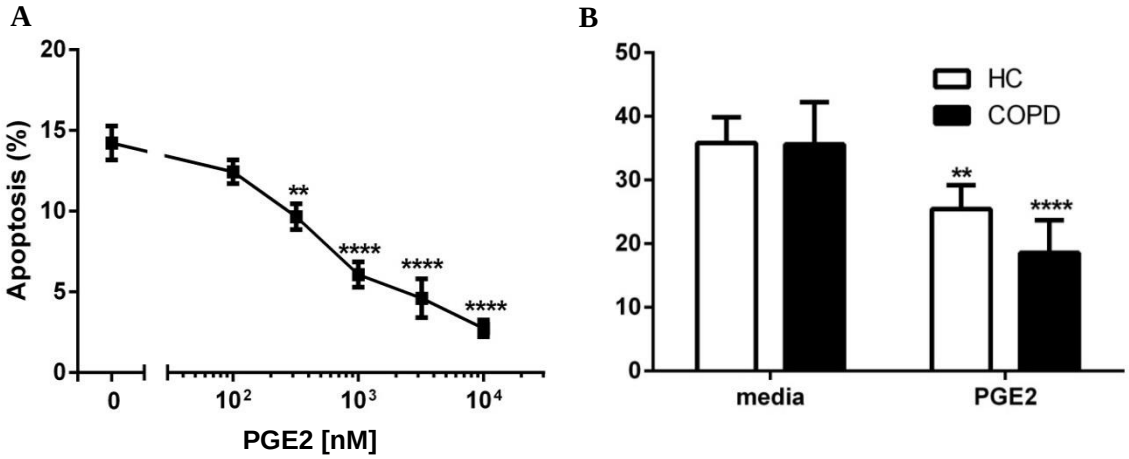
PGE2 is an important agonist at sites of inflammation, with physiological concentrations in the lung to be estimated in the micromolar range, and which can be further increased in the sputum of COPD smokers (Profita *et al.*, 2010). In the same study, PGE2 concentrations correlated with increased neutrophil numbers, which is consistent with diverse studies, where PGE2 or its analogues inhibited apoptosis in PMN (Rossi *et al.*, 1995; Ottonello *et al.*, 1998; Walker *et al.*, 1997; Davern *et al.*, 1999; Ward *et al.*, 2002).

PGE2 has four cell surface receptors: EP1, EP2, EP3 and EP4, by which it mediates its pleiotropic effects (Sugimoto, Narumiya, 2007). Cellular responses of EP1 and EP3 receptor engagement are thought to be mediated by the stimulation of calcium mobilisation or inhibition of adenylyl cyclase (AC) in Chinese hamster ovary cells, respectively (Sugimoto *et al.*, 1992; Sugimoto, Narumiya, 2007). Both receptors have been shown to engage cell death in diverse cell types (Shimamura *et al.*, 2013; Ikeda-Matsuo *et al.*, 2011; Kovarova, Koller, 2014). In contrast, stimulation of EP2 and EP4 activates AC and thereby induces the formation of cAMP (Sugimoto, Narumiya, 2007), which is the main ligand of PKA. EP2 and EP4 are both thought to elicit pro-survival responses through the activation of AC and resulting increases in cAMP. However, EP2 and EP4 can differentially regulate cellular responses and a loss of one receptor can only partly be compensated for by the other receptor.

In PMN, expression of *PTGER2* (EP2), *PTGER3* (EP3) and *PTGER4* (EP4), but not *PTGER1* (EP1), have previously been detected (Yamane *et al.* 2000). The expression of EP receptors can be regulated by inflammatory stimuli, such as LPS (Arakawa *et al.*, 1996; Pavlovic *et al.*, 2006; Katsuyama *et al.*, 1998; Ikegami *et al.*, 2001). Cigarette smoke extract treatment increased EP2 and EP4 levels at early and late timepoints in PMN, as determined by qPCR and western blotting (Profita *et al.*, 2010). Moreover, neutrophilia correlated to increased neutrophilic expression of the EP2 receptor in asthma patients (Corrigan *et al.*, 2012). Additionally, the EP4 receptor is implicated in the PKA-dependent mediation of the anti-inflammatory PGE2 responses for chronic inflammatory conditions of the airways (Birrell *et al.*, 2015). However, the role of the distinct receptors on PGE2 induced neutrophil survival has so far not been examined in detail. Therefore, here the role of PGE2 and the specific involvement of receptor subtypes in PMN survival were examined.

3.1.1. PGE2: Regulation of PMN Survival by Prostaglandin Signalling.

The effect of PGE2 on PMN survival was initially confirmed using PMN isolated from the blood of healthy volunteers (see chapter 2.1.3.). Treatment of PMN with a range of PGE2 concentrations (100 nM – 10 μM) extended PMN lifespan in a dose dependent manner at 4 hours, reaching statistical significance at 0.3 μM (**Fig. 8A**). This is greater than 10-fold in



C

Demographical data of chronic obstructive pulmonary disease patients. Data are presented as mean ± SEM or total number.

Subjects (n)	9
Age (yrs)	**66 ± 1.5**
M/F (n)	**7/2**
Exacerbations in year before donation	**1.8 ± 0.5**
FEV1 (litres)	**1.6 ± 0.1**
FEV1 (%)	**55.2 ± 4.2**
Smoking habits (SM/EX)	**2/7**
Pack years	**45.5 ± 6.8**

Figure 8: ***PGE2 is a Potent Stimulus of PMN Survival in Healthy Control and COPD Neutrophils.*** Percoll purified PMN (Panel **B**) were further refined by negative magnetic selection (Panel **A**) with a custom antibody cocktail (purity > 97 %). PMN were incubated with varying concentrations of PGE2 (0.01 - 10 μM), as indicated. PGE2 treatment dose-dependently decreased apoptosis (Panels **A**, **B**) at 4 hours post inoculation. 10 μM PGE2 exerted a greater effect on PMN from COPD patients than in healthy controls (Panel **B**). Ki values (nM) of PGE2 at prostaglandin receptors are displayed (Panel **C**). Data shown (Panels **A, B**) are mean ± SEM of 3 independent experiments with cells isolated from distinct volunteers, as described in chapter 21.3. Cellular morphology was assessed by light microscopy based on chromatin cleavage and condensation. Representative images are displayed in **Figure 2B**. Statistical analysis was performed through One Way ANOVA with Dunnet's posttest (Panel **A**) or Two Way RM ANOVA with Sidak's posttest (Panel **B**). Asterisks (*****) denote significant differences to untreated conditions. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##), $p<0.001$ (***/###) and $p<0.0001$ (****/####). Abbreviations: HC – healthy control.

excess of the Ki (0.85 – 20 nM) for PGE2 towards its receptors EP1, EP2, EP3 and EP4 (**Table 5**). Additionally, PMN were isolated through Percoll density centrifugation from the blood of COPD patients and healthy control patients. PMN apoptosis was delayed in both COPD patients and healthy controls at 4 hours (**Fig. 8B**). In COPD patient PMN, 10 μM PGE2 increased PMN survival with greater potency than in healthy control PMN. Consequently, potential influences that might cause the lower than expected efficacy of PGE2 in the nanomolar concentration range were investigated. Initially, it was hypothesised that the residual amounts of solvent in the reagents might exert a general cytotoxic effect on PMN, masking the pro survival effect of PGE2.

Therefore, highly purified PMN were incubated with increasing amounts of the solvent dimethyl sulfoxide (DMSO), and the effects of DMSO on PMN apoptosis were investigated. Solvent concentrations lower than or equal to 0.1 % DMSO did not increase apoptosis (**Fig. 9**). A small, but significant increase in PMN apoptosis (2.5 %) was induced by the highest concentration tested (1 % DMSO). The highest concentration of solvent used in any PMN assay did not exceed 0.02 %, indicating that the hypothesized interference by DMSO is negligible in this study.

3.1.1.1. Effect of Metabolic Degradation of PGE2 on PMN Survival.

There is also a theoretical possibility that PGE2 might be degraded to PGF2α or 15-keto PGE2 in the cell culture medium (**Fig. 3**; Polet, Levine, 1975), comparable to its breakdown *in vivo* (Hamberg, Samuelssen, 1971). The key metabolic enzymes regulating PGE2 concentrations are Carbonyl reductases (CBR) and Hydroxyprostaglandin dehydrogenase 15-(NAD) (15-PGDH), which respectively mediate the reduction and oxidation of PGE2 to PGF2α and 15-keto PGE2 (Cho *et al.*, 2006; Nishigaki *et al.*, 1996).

PGF2α was previously shown to delay PMN apoptosis at 10 μM in PMN (Ward *et al.*, 2002). While PGF2α shows affinity for various PGE2 receptors (**Table 5**), 15-keto PGE2 was long thought to be biologically inactive, since it has high Ki values for EP2 and EP4 receptors only (**Table 5**; Nishigaki *et al.*, 1996). However, 15-keto PGE2 was recently shown to act as an intracellular PPARγ ligand (Lu *et al.*, 2013) and it induced low levels of human macrophage apoptosis *in vitro* (Chinetti *et al.*, 1998).

Therefore, it was theorized that the need for high concentrations of PGE2 to mediate PMN survival may be due to PGE2 degradation in cell culture medium and through the additional engagement of the FP receptor subtype. Degradation of PGE2 to 15-keto PGE2 might activate pro-apoptotic PPARγ signalling, and thus further mask the pro-survival effect of PGE2.

Initially, the focus of this investigation was PGF2α, because of its high affinity towards prostaglandin receptors. Here, it was aimed to determine, whether PGF2α would be able to affect early PMN survival in a concentration-dependent manner. Therefore, highly pure PMN

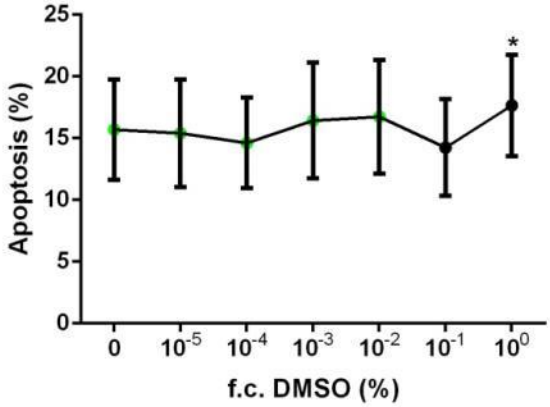


Figure 9: ***Influence of the Solvent DMSO on PMN Apoptosis.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). PMN were incubated with varying concentrations of DMSO (0.00001 – 1 %). 0.00001 – 0.1 % DMSO did not significantly increase apoptosis, while the highest concentration used (1 %) significantly increased PMN apoptosis by a small amount. Statistical analysis was performed through One Way RM ANOVA with Dunnett's posttest. Data shown are mean ± SEM of 4 independent experiments, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. ***** denote significant differences to the untreated control. Results were considered to be statistically significant for $p<0.05$ (*).

Table 5: Affinity (K_i) of PGE2 Metabolic Products for Human Prostanoid Receptors.

Ki values (nM)									
Ligands which displaced over 50% of their respective radioligand at 10 µM									
Ligands	**DP**	**IP**	**TP**	**FP**	**EP1**	**EP2**	**EP3**	**EP4**	**Reference**
PGE2	*	*	*	100 (73 - 140)	20 (15 - 26)	12 (9.2 - 15)	0.85 (0.69 - 1.1)	1.9 (1.5 - 2.5)	Kiriyama *et al.*, 1997
PGF2 α	*	*	*	3.4 (2.8 - 4.2)	1300	*	75 (53 – 110)	*	Kiriyama *et al.*, 1997
dmPGE 2	*	*	*	350 (250 – 480)	*	17 (13-23)	1.9 (1.5 – 2.5)	43 (32 – 58)	Kiriyama *et al.*, 1997
15-keto PGE2	N/K	N/K	N/K	N/K	*	2600	*	15000	Nishigaki *et al.*, 1996

Ligands that did not displace over 50 % of their respective radioligand at 10 µM (*). Abbreviations: DP – DP receptor; IP – IP receptor; TP – TP receptor; FP – FP receptor; N/K – not known.

were incubated with increasing concentrations of PGF2α and PGE2 for comparison, for 4 hours following which PMN apoptosis was assessed. In a non-sigmoidal dose response for concentrations up to 1 μM, PGF2α increased PMN apoptosis hyperbolically, to then steeply drop off at 10 μM to induce comparable levels of PMN survival to 10 μM PGE2 (**Fig. 10A**).

PGF2α shows a high affinity for the FP receptor, which is its main receptor (**Table 5**). Therefore, a partial degradation of PGE2 to PGF2α might exert their action through the high affinity FP receptor. To determine the effect of FP receptor inhibition on PMN survival, highly pure PMN were preincubated for 15 minutes with the irreversible FP receptor antagonist AL 8810, before the addition of varying concentrations of PGE2 for 4 hours. In a control assay, AL 8810 alone did not significantly increase PMN apoptosis, even at the highest concentration used (10 μM; **Fig. 10B**). However, treatment with AL 8810 blocked PGE2 induced PMN survival (**Fig. 10B**, **C**). At an agonist concentration of 0.1 μM, PGE2 induced survival was significantly blocked by treatment with 10 μM AL 8810 (**Fig. 10C**).

Due to the reasons outlined above, it was hypothesised that degradation of PGE2 by 15-PGDH might target nuclear PPARγ in PMN, and could thus induce alterations in PMN survival. To test this hypothesis, highly pure PMN were incubated with increasing concentrations of the selective PPARγ agonist rosiglitazone or PGE2 for 4 hours. Conversely, rosiglitazone initially increased PMN apoptosis at concentrations well below its EC50 for PPARγ (EC50= 60 nM). This increase was dose-dependently reversed by higher agonist concentrations (0.1 – 10 μM; **Fig. 11A**), and thereby mirrored PGE2-incuded survival for these concentrations. To further elucidate the role of PPARγ on PMN survival, the competitive PPARγ antagonist GW 9662 was used. GW 9662 alone did not significantly alter PMN survival at any of the concentrations used (**Fig. 11B**). Thereafter, highly pure PMN were preincubated with GW 9662 for 15 minutes and subsequently cultured in presence of varying concentrations of PGE2 for 4 hours. Interestingly, PGE2-induced survival at 0.1 μM was significantly blocked by treatment with 1 μM GW 9662 (**Fig. 11C**), while GW 9662 did not significantly block PGE2 survival induced at higher concentrations of the agonist.

Thereafter, the hypothesis was further explored that the efficacy of PGE2 at supra-Ki concentrations only was due to a decrease in biologically active PGE2 concentrations caused by PGE2 metabolism. 16,16 dimethyl PGE2 (dmPGE2) is a PGE2 analogue with increased resistance towards PGDH metabolism. It competitively inhibits 15-PGDH, without agonistic properties (Ohno *et al.*, 1978). In fact, the half-life of dmPGE2 is prolonged *in vivo* (Ohno *et al.*, 1978). Comparable to PGE2, dmPGE2 acts as an agonist on most PGE2 receptors (**Table 5**; Kiriyama *et al.*, 1997). Furthermore, the reagent was shown to mediate physiological PGE2 responses with increased potency in comparison to PGE2 (Robert *et al.*, 1976).

To assess the influence of PGE2 stability on PMN survival, highly pure PMN were incubated with the metabolically stable PGE2 analogue dmPGE2 in the presence of varying concentrations

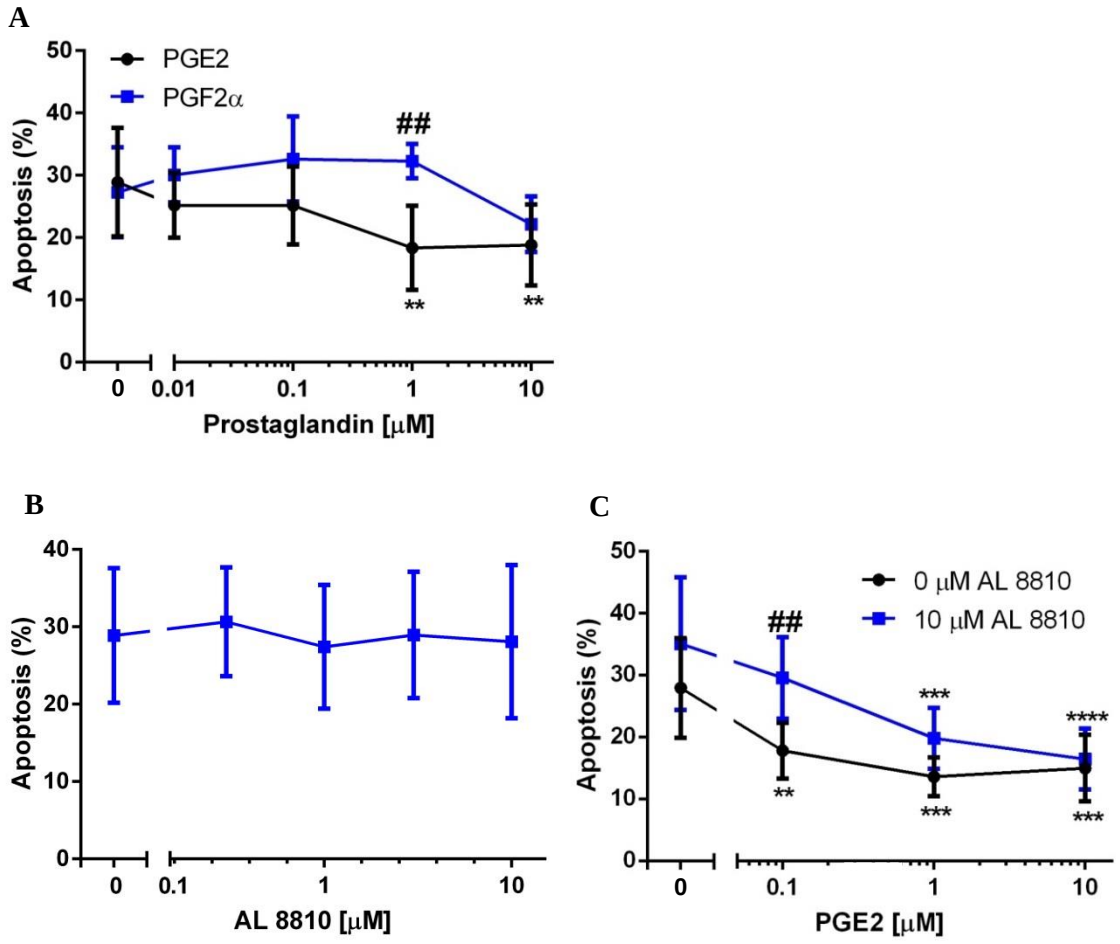


Figure 10: ***PMN Survival Induced by High PGE2 Concentrations is not Triggered by PGE2 Metabolism to PGF2α***. Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). Ultrapure PMN were cultured with 0.01 – 10 μM PGE2 (Panel **A, C**) or PGF2α (Panel **A**). In some experiments, PMN were additionally preincubated with the FP receptor antagonist AL 8810 for 15 minutes (Panels **B, C**). PGE2 induced PMN survival dose dependently (Panel **A**), while PGF2α significantly increased PMN apoptosis at concentrations ≤ 1 μM, but induced PMN survival at 10 μM (Panel **A**). Statistical analysis was performed through Two Way RM ANOVA with Sidak's post tests (Panels **A, C**), or One Way RM ANOVA with Dunnett's posttests (Panel **B**). Data shown are mean ± SEM of 3 (Panels **A, B**) or 5 (Panel **C**) independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. ***** denote differences to control conditions. **#** indicate differences to the PGE2 control curve. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##), $p<0.001$ (***/###) and $p<0.0001$ (****/####).

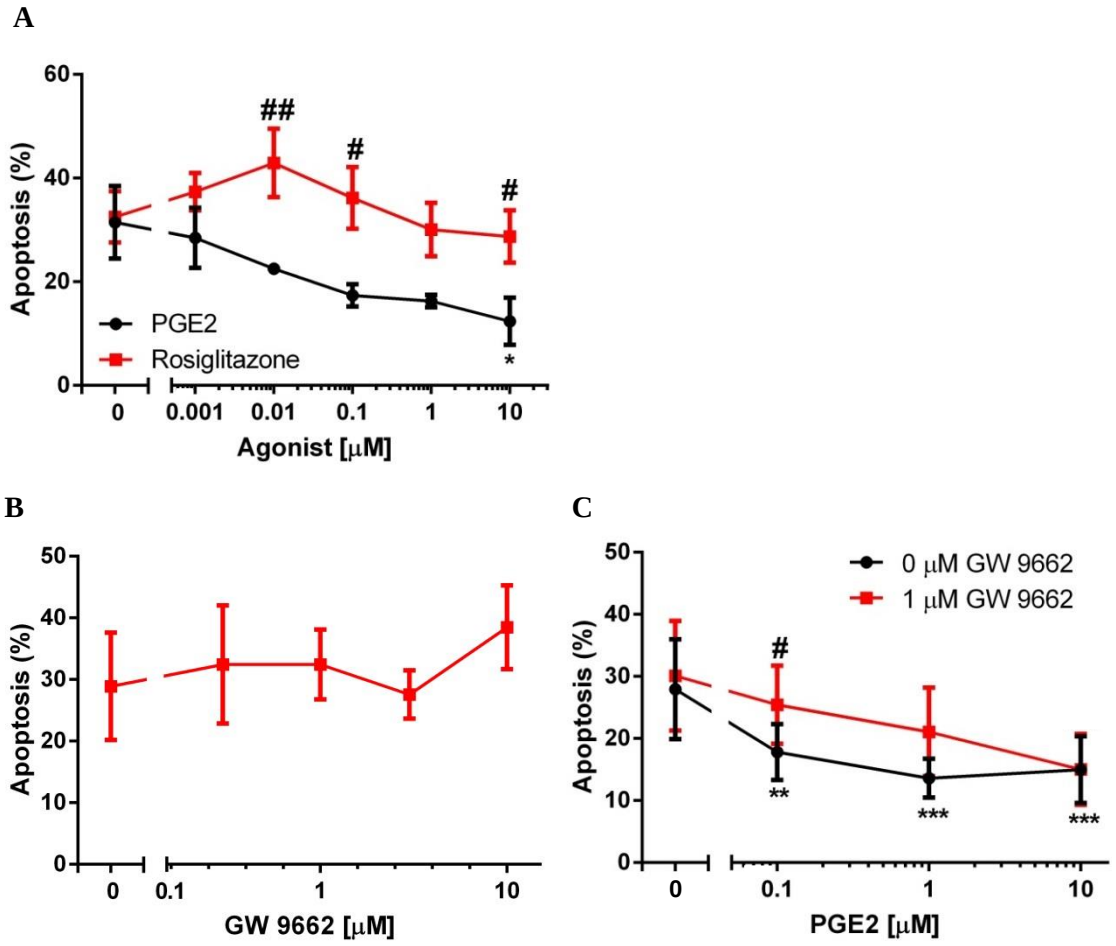


Figure 11: ***Off Target Activity of High PGE2 Concentrations Prompts PMN Survival Extension Through Engagement of the PPARγ Receptor.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). Ultrapure PMN were preincubated for 15 minutes in presence or absence of the PPARγ antagonist GW 9662 (Panels **C, D**). Thereafter, 0.001 - 10 μM PGE2 (Panel **B**, **C**; as indicated), the PPARγ agonist rosiglitazone (Panel **A**) or the antagonist alone (Panel **B**) were added for 4 hours. Rosiglitazone increased PMN apoptosis until a concentration of 0.01 μM, but then mirrored the PGE2 survival curve at higher concentrations (Panel **A**). GW 9662 alone did not significantly increase PGE2 induced PMN survival (Panel **B**), although GW 9662 showed a tendency towards increased apoptosis at 10 μM. However, GW 9662 specifically increased PGE2 induced survival at 0.1 μM agonist concentration, consistent with a potential off target effect of PGE2 at the PPARγ receptor. Statistical analysis was performed through Two Way ANOVA with Sidak's posttest (Panels A, C) or One-Way ANOVA with Dunnett's posttest (Panel **B**). Data shown are mean ± SEM of 3 (Panel **B**), 4 (Panel **A**) or 5 (Panel **C**) independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. ***** denote differences to control conditions. **#** indicate differences to the PGE2 control curve. Cellular morphology was assessed by light microscopy. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##) and $p<0.001$ (***/###).

of PGE2 or dmPGE2 (as indicated) for 4 hours (**Fig. 12A, B**) and 20 hours (**Fig. 12B**). It was found that the efficacy of dmPGE2 at inducing PMN survival was mostly similar to PGE2, where dmPGE2 reached statistical significance at 1 μM and PGE2 at 0.1 μM at 4 hours (**Fig. 12A**). In a separate assay on the temporal effect of the agonists, there was no difference between PMN survival induced by 1 μM PGE2 and 1 μM dmPGE2 at 4 hours (**Fig. 12B**). However, at 20 hours, PGE2 was unable to sustain PMN survival, whereas dmPGE2 significantly decreased PMN apoptosis at 20 hours.

3.1.2. PGE2 Receptors: Involvement of the EP2 and EP4 Receptor Subtypes in PGE2 Signalling.

3.1.2.1. Expression of EP Receptor mRNA in PMN.

Prostaglandin receptors can be classified based on their respective PGE2 affinity: EP1 and EP2 have a low affinity to PGE2, whereas EP3 and EP4 are classified as high-affinity receptors due to their respective Ki values (**Table 6**). The aim of this chapter was to identify the receptor subtypes that mediate the pro-survival effects of PGE2. Firstly, the mRNA expression of the PGE2 receptor subtypes in PMN was investigated by RT-PCR. For this, total RNA was extracted from highly pure PMN and reverse transcribed, as per chapter 2.3. Sequence-verified RT-PCR primers (kind gift of Dr. Linda Kay) were previously used by Kay *et al.* (2013). Densitometric RT-PCR analysis, as described in chapter 2.3.3., revealed that EP4 receptors and low levels of mRNA for EP2 are expressed in PMN (**Fig. 13A, B**). mRNA expression of EP1 and EP3 receptors was not detected.

3.1.2.2. Effect of EP Receptor Agonism on PMN Survival.

To demonstrate the involvement of specific EP receptor subtypes in PMN survival, highly pure PMN were incubated with increasing concentrations (0.1 μM – 10 μM) of PGE2 or four distinct PGE2 receptor agonists: the EP1 receptor agonist 17-phenyl trinor PGE2, the EP2 receptor agonist butaprost, the EP3 receptor agonist sulprostone and the EP4 receptor agonist L-902,688. In this context, receptor expression of both EP2 and EP4 were previously demonstrated (**Fig. 13**), whereas EP1 and EP3 receptor expression was not detected on the mRNA level. However, EP1 and EP3 might possess high protein stability, and might still be expressed on the protein level.

Here, incubation with the EP1 agonist 17-phenyl-trinor-PGE2 significantly reduced PMN survival at an agonist concentration of 1 μM, where the agonist reached its maximum efficiency (**Fig. 14A**). The EP1 agonist only induced a small amount of PMN survival for 1 concentration tested (1 μM), whereas the PGE2 control curve dose-dependently induced survival in PMN at the three highest concentrations (1-10 μM).

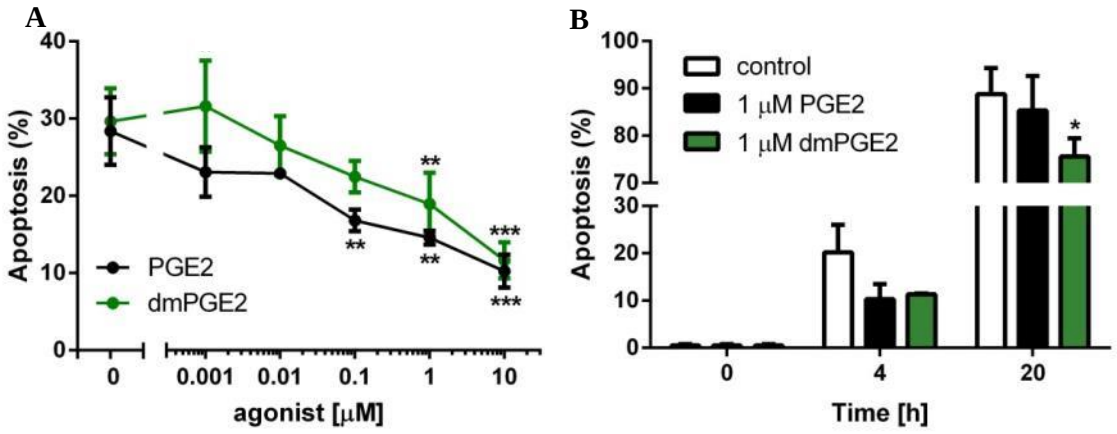


Figure 12: ***Early Effect of PGE2 is not Dependent on Low Metabolic Stability.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). Ultrapure PMN were incubated for 4 hours (Panels **A, B**) or 20 hours (Panel **B**) with varying concentrations (as indicated) of PGE2, or its analogue dmPGE2. PGE2 and dmPGE2 significantly and dose-dependently enhanced PMN survival at 4 hours (Panel **A**). However, dmPGE2 was significantly less effective at inducing PMN survival at 0.001 µM of the agonist, compared to PGE2. Moreover, with 1 µM PGE2 or dmPGE2, there was no significant difference between the agonists at 4 hours, but dmPGE2 retained its influence on PMN survival at 20 hours (Panel **B**). Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. * denote differences to control conditions. # indicate differences to the PGE2 control curve. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##) and $p<0.001$ (***/###).

Table 6: Affinity (K_i) of PGE2, Agonists and Antagonists for Human Prostanoid Receptors.

Ki values (nM)									
Ligands which displaced over 50% of their respective radioligand at 10 µM									
Ligands	**DP**	**IP**	**TP**	**FP**	**EP1**	**EP2**	**EP3**	**EP4**	**Reference**
PGE2	*	*	*	100 (73 - 140)	20 (15 - 26)	12 (9.2 - 15)	0.85 (0.69 - 1.1)	1.9 (1.5 - 2.5)	Kiriyama *et al.*, 1997
17 phenyl trinor PGE2	*	*	*	60 (47 - 77)	14 (11 - 18)	*	3.7 (2.8 - 4.9)	1000	Kiriyama *et al.*, 1997
(R)-Butaprost (free acid)	*	*	*	*	*	110 (83 - 140)	*	*	Kiriyama *et al.*, 1997
Sulprostone	*	*	*	580 (360 - 930)	21 (17 - 25)	*	0.60 (0.44 - 0.81)	*	Kiriyama *et al.*, 1997
L 902,688	N/K	N/K	N/K	N/K	>1500	>1500	>1500	0.38	Young *et al.*, 2004
PF 04418948	N/K	N/K	N/K	N/K	N/K	1.3 (K_B)	N/K	N/K	Birrell *et al.*, 2013; Af Forselles *et al.*, 2011
GW627368X	>5000	>5000	158	>5000	>5000	>5000	>5000	100	Wilson *et al.*,2006

Ligands that did not displace over 50 % of their respective radioligand at 10 µM (*). Abbreviations: DP – DP receptor; IP – IP receptor; TP – TP receptor; FP – FP receptor; N/K – not known.

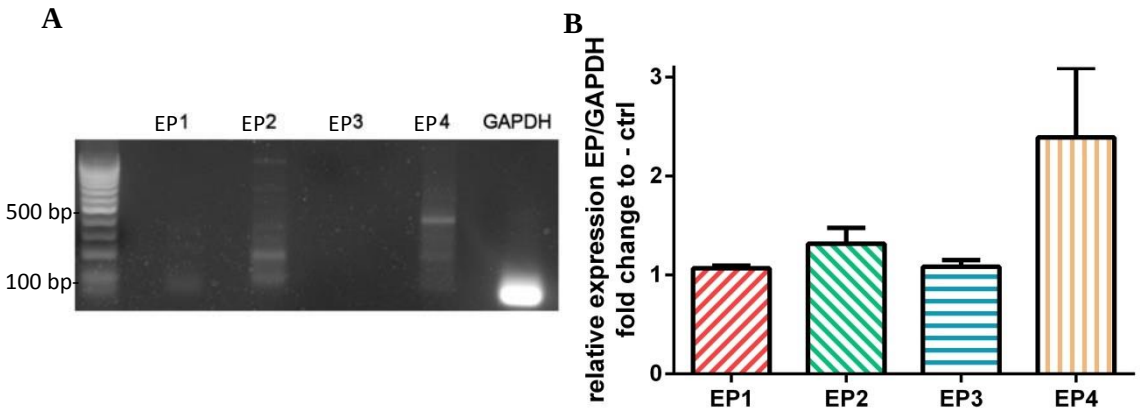


Figure 13: ***Constitutive Expression of Prostaglandin Receptors in PMN.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). mRNA was isolated from PMN by TRI extraction (as described in chapter 2.3.1.). *PTGER2* (EP2) and *PTGER4* (EP4), but not *PTGER1* (EP1) and *PTGER3* (EP3) were transcribed in ultrapure PMN through RT-PCR (Panel **A, B**). Expected band sizes for PCR amplification products (Panel **A**) of the PCR primers (described in Appendix 4) were as follows: *PTGER1* (EP1) – 149 bp, *PTGER2* (EP2) – 419 bp, *PTGER3* (EP3) – 837 bp, *PTGER4* (EP4) – 434 bp. Data shown are mean ± SEM of 4 independent experiments (Panel **B**), as described in chapter 2.5. Densitometry was performed using the ImageJ software.

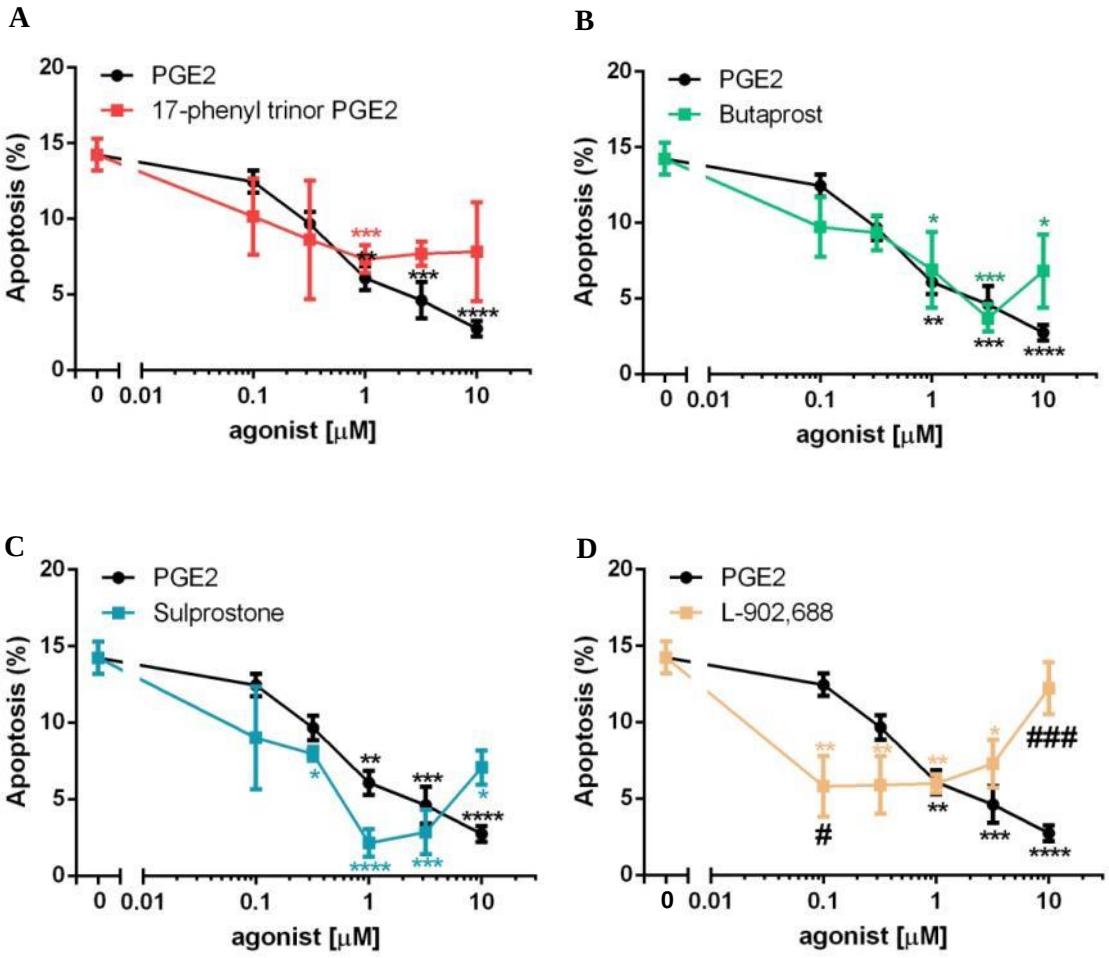


Figure 14: ***Agonism of Prostaglandin Receptors EP2, EP3 and EP4 Induces PMN Survival.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). Ultrapure PMN were incubated with 0.1 – 10 μM PGE2, the EP1 agonist 17-phenyl trinor PGE2 (Panel **A**), the EP2 agonist butaprost (Panel **B**), the EP3 agonist sulprostone (Panel **C**) or the EP4 agonist L-902,688 (Panel **D**). 17-phenyl trinor PGE2 moderately delayed PMN survival with a maximal biological response below 1 μM (Panel **A**). Butaprost enhanced PMN survival in a dose-dependent manner, comparable to PGE2 survival (Panel **B**). Sulprostone induced moderate PMN survival (Panel **C**). L-902,688 acted as a partial agonist at concentrations below 1 μM (Panel **D**). Statistical analysis was performed through Two Way ANOVA with Sidak's posttests. Data shown are mean ± SEM of 4 independent experiments, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. ***** denote significant differences to untreated controls and **#** indicate differences between PGE2 and treatment with the respective agonist. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##), $p<0.001$ (***/###) and $p<0.0001$ (****/####).

The EP2 receptor agonist butaprost (free acid) enhanced PMN survival dose-dependently for concentrations up to 3 μM, and had a slightly decreased, but significant effect on PMN survival at 10 μM (**Fig. 14B**). It thereby mirrored the effect of PGE2, which significantly induced survival for concentrations of 1-10 μM.

In the initial phase of the curve, treatment with the EP3 agonist sulprostone shifted the dose response curve on PMN survival to the left, compared to the PGE2 dose-response curve. Sulprostone significantly inhibited neutrophil apoptosis at 300 nM, but was without further effect on apoptosis at concentrations greater than 1 μM (**Fig. 14C**).

The EP4 agonist L-902,688 was extremely potent and resulted in a strong left-shift compared to the PGE2 curve (**Fig. 14D**). L-902,688 significantly increased PMN survival at 0.1 – 3 μM and its maximal biological response was observed at the lowest agonist concentration used (0.1 μM). Interestingly, the efficacy of L-902,688 deteriorated with increasing agonist concentrations from 1 – 10 μM. A comparable increase with high concentrations was observed for all agonists (**Fig. 14B-D**), apart from 17-phenyl trinor PGE2 (**Fig. 14A**).

3.1.2.3. Effect of EP2 and EP4 Receptor Antagonism on PMN Survival.

Antagonism of EP3 receptors in human PMN has previously been characterised and a non-apoptotic form of cell death was found (Liu *et al.*, 2005; Liu *et al.*, 2007). Therefore, it was chosen to further investigate the involvement of EP2 and EP4 receptors in PGE2 survival. Here, it was aimed to confirm the findings of the agonist studies presented above; by using selective receptor antagonists to block PGE2 induced survival.

For this end, the EP2-selective antagonist PF-04418948 and the EP4-selective antagonist GW 627368X were incubated with highly pure PMN for 4 hours. Incubation with 3 – 300 nM of either antagonist alone or in combination did not significantly increase constitutive PMN apoptosis (**Fig. 15**).

To determine whether PGE2-induced survival was mediated through the EP2 receptor, highly pure PMN were pre-incubated for 15 minutes with the indicated amount of PF-04418948. Thereafter, a range of PGE2 concentrations (3 nM – 10 μM; as indicated; **Fig. 16A, B**) or a range of butaprost concentrations (3 nM – 10 μM; **Fig. 16C**) was added, and incubated for 4 hours. As expected, PGE2 significantly induced PMN survival at 3 μM and 10 μM at 4 hours (**Fig. 16A**). PF-04418948 alone was without effect on constitutive apoptosis. However, 300 nM of the EP2 antagonist blocked PGE2-induced PMN survival (at concentrations of 30 nM to 3 μM PGE2) and increased apoptosis by a mean of 8.3 % (± 0.2 SEM) in comparison to the PGE2 curve. Similarly, a higher concentration of PF-04418948 (10 μM) increased PMN apoptosis by 8.0 % (± 0.5 SEM). However, statistical significance was only achieved for 1 μM PGE2 (**Fig. 16B**). PF-04418948 [0.3 μM] significantly blocked survival induced by the EP2 agonist butaprost at 3 μM (**Fig. 16C**).

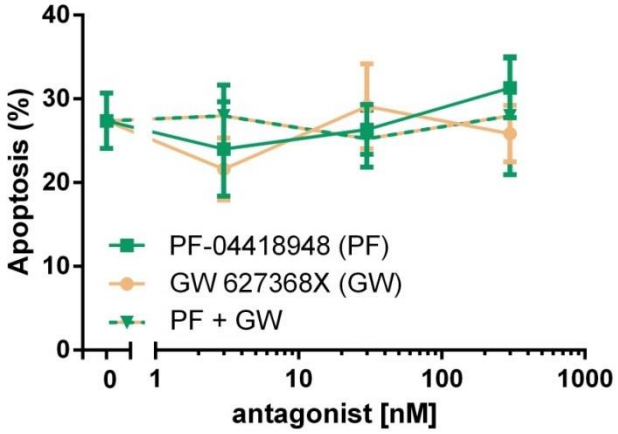


Figure 15: ***Effect of EP2 Receptor Antagonist PF-04418948 and EP4-Receptor Antagonist GW 627368X on Basal PMN Survival.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). Ultrapure PMN were incubated in presence or absence of 3 – 300 nM PF-04418948, GW 627368X or both for 4 hours. Incubation with PF-04418948 and GW 627368X alone or in combination did not significantly influence PMN survival at any of the concentrations tested. Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests. Data shown are mean ± SEM of 4 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. ***** denote significant differences to untreated conditions and **#** indicate differences between treatments with different antagonists. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##), $p<0.001$ (***/###) and $p<0.0001$ (****/####).

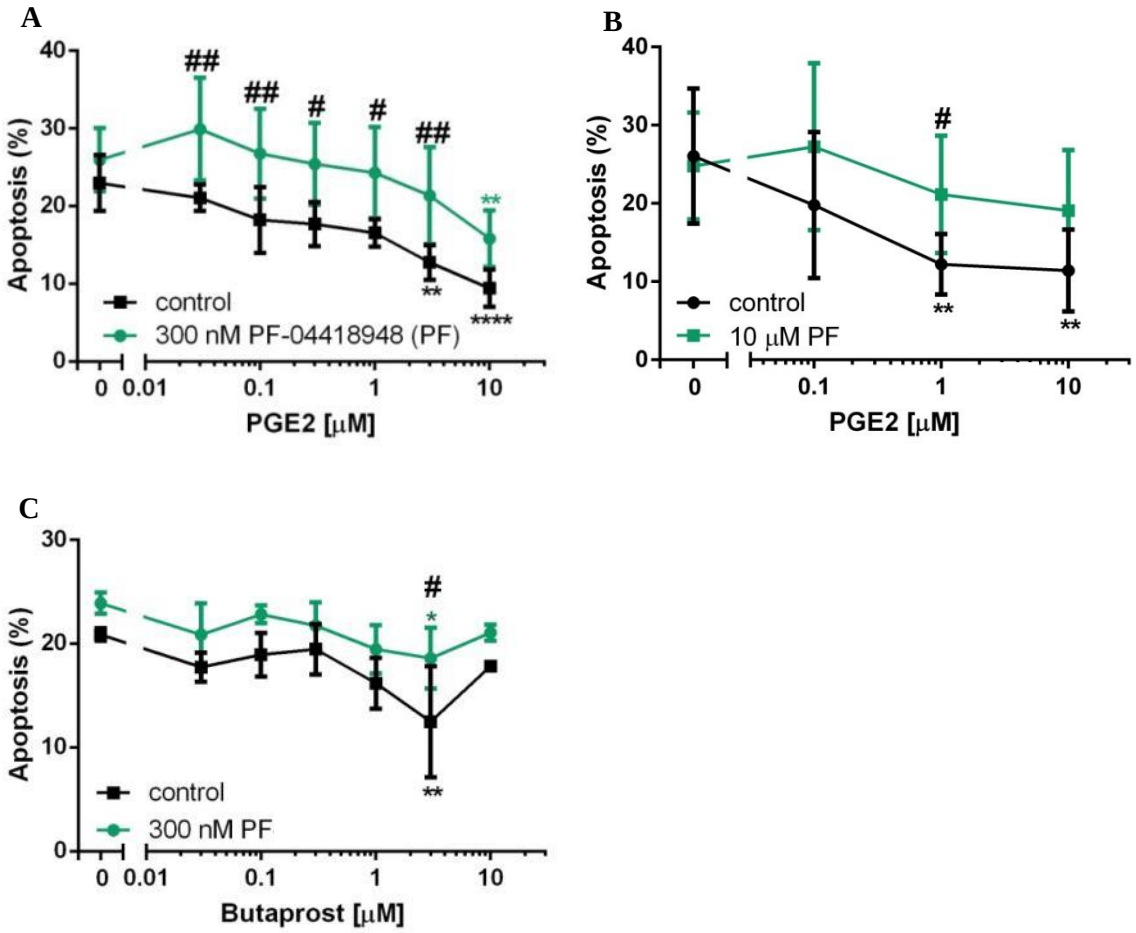


Figure 16: ***EP2 Receptor Antagonist PF-04418948 Blocks Agonist Induced PMN Survival.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). Ultrapure PMN were preincubated for 15 minutes in presence or absence of 300 nM (Panels **A**, **C**) or 10 μM (Panel **B**) of the EP2 antagonist PF-04418948. Thereafter, varying concentrations of PGE2 (Panels **A**, **B**) or the EP2 agonist (R)-Butaprost (Panel **C**) were added to treatment conditions for a further 4 hours. PGE2 significantly delayed PMN survival. 0.3 μM PF-04418948 partially, but irreversibly, decreased PGE2 survival additional to constitutively increased PMN survival with PF-04418948 alone (Panel **A**). Incubation with PF-04418948 resulted in a complete and non-competitive inhibition of the effects of low PGE2 levels (< 1 μM). Treatment with PF-04418948 for high PGE2 concentrations (> 1 μM) only partially inhibits the survival effects of PGE2 (Panel **A**). The extent of PGE2 inhibition was not further increased by treatment with 10 μM PF-04418948 (Panel **B**). PF-04418948 enhanced PMN apoptosis, but only blocked (R)-Butaprost induced survival at 3 μM (R)-Butaprost (Panel **C**). Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests. Data shown are mean ± SEM of 3 independent experiments (Panels **A**, **C**) or 4 independent experiments (Panels **B**) with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. ***** denote significant differences to untreated conditions and # indicate differences to the agonist control curve. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##), $p<0.001$ (***/###) and $p<0.0001$ (****/####).

To determine, whether PGE2 induced survival was mediated through the EP4 receptor subtype, highly pure PMN were pre-incubated for 15 minutes with the indicated amount of GW 627368X. Thereafter, a range of PGE2 concentrations (3 nM – 10 μM; as indicated; **Fig. 17A, B**) or a range of L-902,688 concentrations (3 nM – 10 μM; **Fig. 17C**) was added, and incubated for 4 hours.

The EP4 antagonist GW 627368X alone did not significantly increase constitutive survival (**Fig. 17A**). However, only one concentration of PGE2 induced survival (0.1 μM) was significantly antagonised by 300 nM GW 627368X, which only increased PMN apoptosis by less 6.7 %. However, also by using a higher antagonist concentration of 1 μM, only PGE2 induced survival at 100 nM was significantly blocked, with a mean percentage difference at higher concentrations of only 2.52 % ± 0.98 (**Fig. 17B**). L-902,688 at concentrations of between 10 nM and 1 μM significantly induced PMN survival, which was not blocked by treatment with 0.3 μM GW 627368X (**Fig. 17C**).

Co-treatment of both the EP2 and EP4 antagonists for 4 hours did not significantly increase PMN apoptosis for the antagonists alone in highly pure PMN (**Fig. 15**). However, treatment with both antagonists significantly protected PMN from PGE2 induced survival at all tested concentrations (0.1 – 10 μM PGE2) and increased PMN apoptosis by 10.4 % (± 1.5 SEM) in comparison to PGE2 (**Fig. 18A**). A comparison between the effect of 10 μM PF-04418948 and 10 μM of both antagonists on PGE2 induced survival revealed only a very small difference, which was not significant (**Fig. 18B**).

3.2. Evaluating the Influence of LPS on PGE2 Receptor Expression in the Context of COPD.

There is increasing evidence that LPS and PGE2 induced signalling pathways are interrelated, and may have synergistic effects on the mediation of cellular responses in inflammation. An autoregulatory loop for PGE2 has previously been reported in macrophages, where PGE2 treatment downregulated EP4 mRNA expression, but not EP2 mRNA (Ikegami *et al.*, 2001). Moreover, LPS treatment transiently increased EP2 mRNA expression, but downregulated mRNA for EP4 (Ikegami *et al.*, 2001). In the macrophage cell line RAW 264.7, LPS also upregulated mRNA for EP2, but not EP4, while PGE2 downregulated mRNA for both and constitutive expression of EP4 was higher than EP2 (Hubbard *et al.*, 2001). Moreover, LPS-induced Cox2 expression was found to be mediated by the EP4 receptor in macrophages (Pavlovic *et al.*, 2006). Therefore, it was hypothesised that the inflammatory stimuli LPS and PGE2 can modulate EP receptor expression as autoregulatory responses in the control of inflammatory PMN survival.

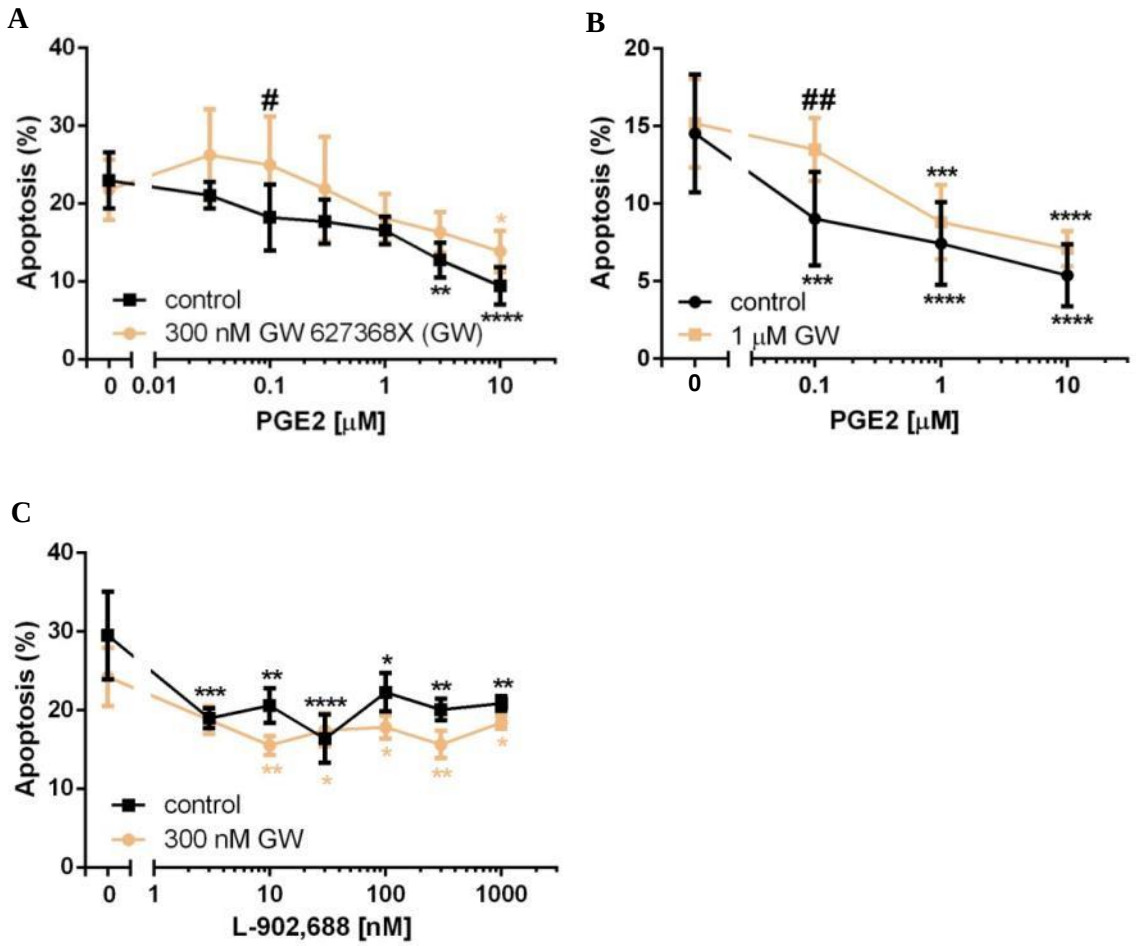


Figure 17: ***Effect of EP4 Receptor Antagonist GW 627368X on Agonist Induced PMN Survival.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). Ultrapure PMN were preincubated for 15 minutes in presence or absence of 300 nM (Panels **A**, **C**) or 10 μM (Panel **B**) of the EP4 antagonist GW 627368X. Thereafter, varying concentrations of PGE2 (Panels **A**, **B**) or the EP4 agonist L-902,688 (Panel **C**) were added to treatment conditions for a further 4 hours. PGE2 significantly delayed PMN survival. Reversal of PGE2 induced PMN survival was observed for concentrations <1 μM PGE2 by incubation with 0.3 μM GW 627368X (Panel **A**). Treatment with GW 627368X for high PGE2 concentrations (> 1 μM) only partially inhibited the survival effects of PGE2. GW 627368X induced blocking of PGE2 survival was not further increased by a higher antagonist concentration of 10 μM (Panel **B**). GW 627368X showed weak intrinsic agonist activity and a potentially partial blockage of L-902,688 induced survival (Panel **C**). Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests. Data shown are mean ± SEM of 3 independent experiments (Panels **A**, **C**) or 5 independent experiments (Panels **B**) with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. ***** denote significant differences to untreated conditions and **#** indicate differences to the agonist control curve. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##), $p<0.001$ (***/###) and $p<0.0001$ (****/####).

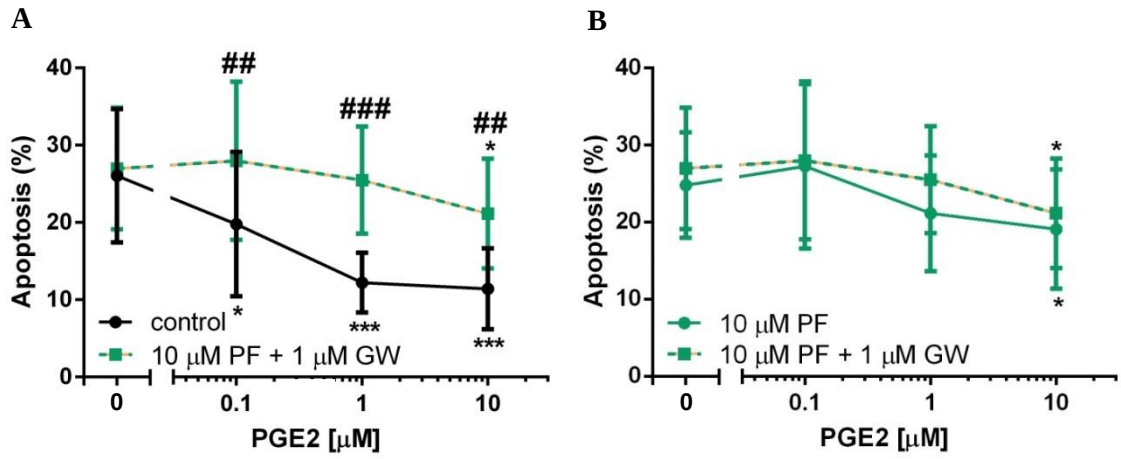


Figure 18: ***Antagonism of the EP2 Receptor is Sufficient to Block PGE2 induced Survival.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). Ultrapure PMN were preincubated for 15 minutes in presence or absence of 10 μM of the EP2 antagonist PF-04418948 and the EP4 antagonist GW 627368X. Thereafter, 0.1 – 10 μM PGE2 were added to treatment conditions for a further 4 hours. Incubation with PF-04418948 and GW 627368X in combination significantly blocked PGE2 induced survival (Panel **A**). However, no significant differences were observed between PGE2 survival blocked with PF-04418948 only and both antagonists (Panel **B**). Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests. Data shown are mean ± SEM of 4 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. ***** denote significant differences to untreated conditions and **#** indicate differences to the PGE2 control curve. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##), $p<0.001$ (***/###) and $p<0.0001$ (****/####).

3.2.1. Induction of EP2 and EP4 Receptor Expression by LPS.

Initially, the effect of LPS on PGE2 signalling and PGE2 receptor expression in PMN was examined. For this, highly pure PMN were incubated with 1 ng/ml LPS, 10 μM PGE2 or both for 4 hours and receptor mRNA transcript levels were examined by RT-PCR (as described in chapter 2.3.). Constitutive mRNA expression for both EP1 and EP3 were undetectable (**Fig. 13**). Moreover, neither EP1, nor EP3 mRNA expression was induced by LPS or PGE2 (**Fig. 19**). In contrast, treatment with LPS induced the expression of EP2 mRNA, from a very low constitutive level at 4 hours in PMN (**Fig. 20A, B**). Similarly, EP4 mRNA was also upregulated at 4 hours by LPS (**Fig. 20A, C**). Neither EP2 nor EP4 were significantly regulated by PGE2, whereas EP4 expression was non-significantly decreased in response to PGE2 (**Fig. 20C**). Moreover, treatment with both LPS and PGE2 increased EP2 and EP4 mRNA transcript levels in comparison to the control (**Fig. 20A-C**).

These findings were confirmed by qPCR, which is a more quantitative assessment of gene expression. The constitutive expression of EP4 was 16.23 ± 8.40 (S.E.M.) times greater than the basal levels of EP2 mRNA (**Fig. 21A**). LPS significantly upregulated EP2 expression over 5 fold (**Fig. 21B**), and over 12 fold in EP4 mRNA expression (**Fig. 21C**). Interestingly, incubation with PGE2 significantly decreased EP4, but not EP2 mRNA expression. Remarkably, LPS induced expression of both EP2 and EP4 mRNA was attenuated by PGE2 treatment.

3.2.2. Functional Consequences of EP2 and EP4 mRNA Regulation in the Context of COPD.

In this chapter, the mRNA expression of EP2 and EP4 in response to LPS was examined in the context of COPD. For this, Percoll pure PMN from healthy control and COPD patients (recruited through the COPD MAP Initiative; **Fig. 8C**) were incubated in the presence of 10 ng/ml LPS for up to 2 hours. mRNA expression for EP2 and EP4 were analysed by qPCR. LPS induced EP2 mRNA expression in a temporal manner up to 2 hours (**Fig. 22B**). At 2 hours, EP2 mRNA expression was significantly enhanced in COPD patient PMN in contrast to healthy control PMN. Similarly, EP4 mRNA expression was increased over 2 hours of LPS treatment, although this was not significant (**Fig. 23B**). However, there was no detectable difference between mRNA expression in COPD and healthy control PMN, as evidenced by the upregulation of EP expression in responsive donors only, independent of patient classification as healthy control or COPD patient (**Fig. 22D; Fig. 23D**).

In a separate assay, Percoll pure PMN from healthy control and COPD patients were incubated in the presence of 10 ng/ml LPS, 10 μM PGE2 or both for 4 hours. mRNA expression for EP2 and EP4 were analysed by qPCR. In this assay, LPS non-significantly increased mRNA expression of EP2 to comparable levels in both COPD and healthy control PMN (**Fig. 22C**). The increased rate of EP2 upregulation by LPS in COPD PMN (**Fig. 22B**) appeared to be

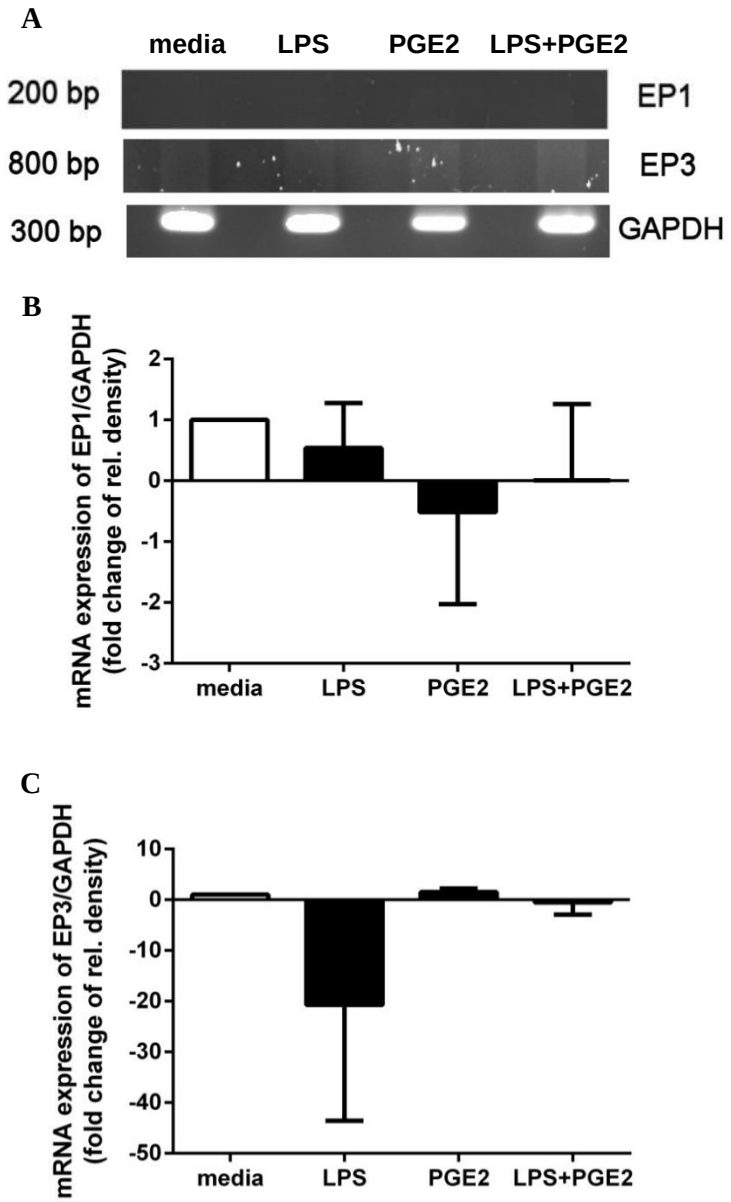


Figure 19: ***LPS Induced PTGER1 and PTGER3 Expression is Attenuated by PGE2.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). mRNA was extracted from PMN directly following the PMN preparation using the TRI method (as described in chapter 2.3.). Total cellular RNA was reverse transcribed into cDNA using random primers. Resulting cDNA was then analysed through densitometry by RT-PCR using primers for *PTGER1* (Panels **B**), *PTGER3* (Panels **C**) and *GAPDH* as a normalisation control. PMN were incubated for 4 hours with LPS (1 ng/ml), PGE2 (10 μM) or both. Neither EP1, nor EP3 were significantly regulated by treatment with LPS or PGE2. Statistical analysis was performed through One Way RM ANOVA with Dunnett's posttests on normalised Ct values (ΔCT), and displayed on graphs for $2^{\Delta CT}$, where applicable. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Results were considered to be statistically significant for $p<0.05$ (*/#).

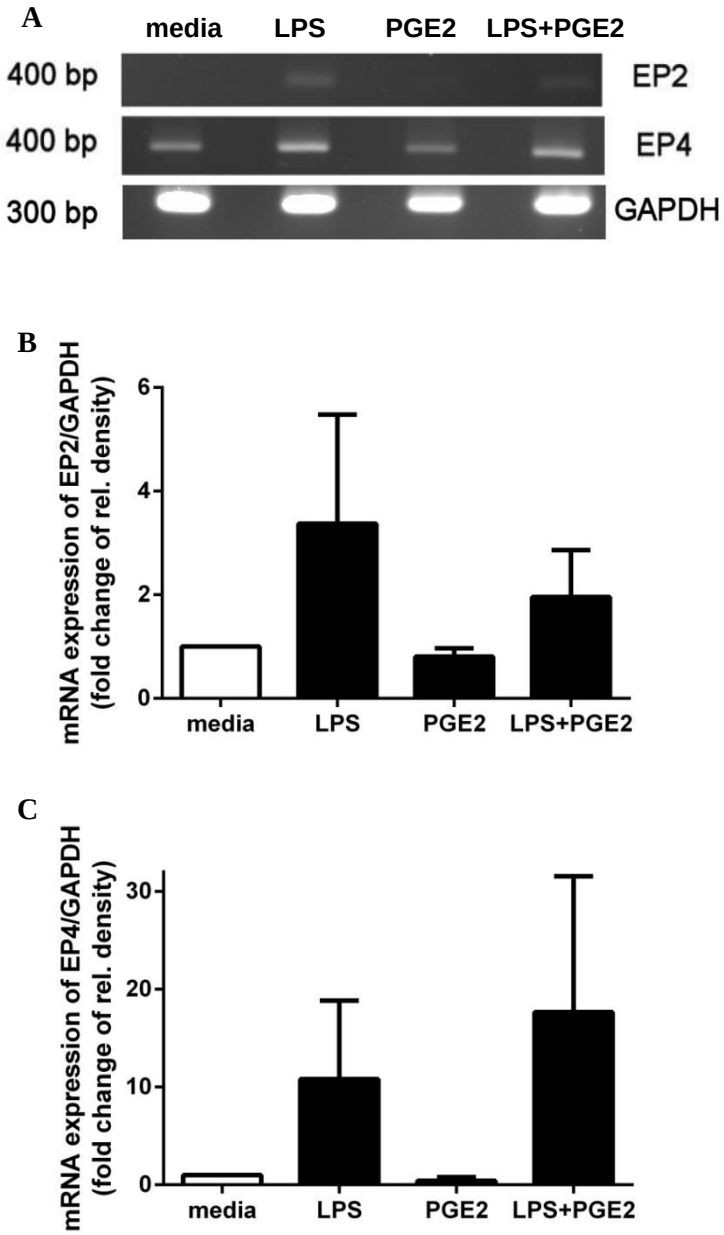


Figure 20: ***Effect of PGE2 and LPS on EP2 and EP4 Expression in PMN.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). mRNA was extracted from PMN directly following the PMN preparation using the TRI method (as described in chapter 2.3.). Total cellular RNA was reverse transcribed into cDNA using random primers. Resulting cDNA was then analysed densitometry through RTPCR using primers for *PTGER2* (EP2; Panel **B**), *PTGER4* (EP4; Panel **C**) and *GAPDH* as a normalisation control. PMN were incubated for 4 hours with LPS (1 ng/ml), PGE2 (10 µM) or both. LPS non-significantly increased EP2 mRNA and EP4 mRNA expression at 4 hours in PMN (Panels **B, C**). PGE2 alone had little effect on expression levels, but potentially reduced LPS-induced EP2 (Panels **B**) mRNA expression levels by RT-PCR. Statistical analysis was performed through One Way RM ANOVA with Dunnett's posttests on normalised Ct values (ΔCT), and displayed on graphs for $2^{\Delta CT}$, where applicable. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Results were considered to be statistically significant for $p<0.05$ (*/#).

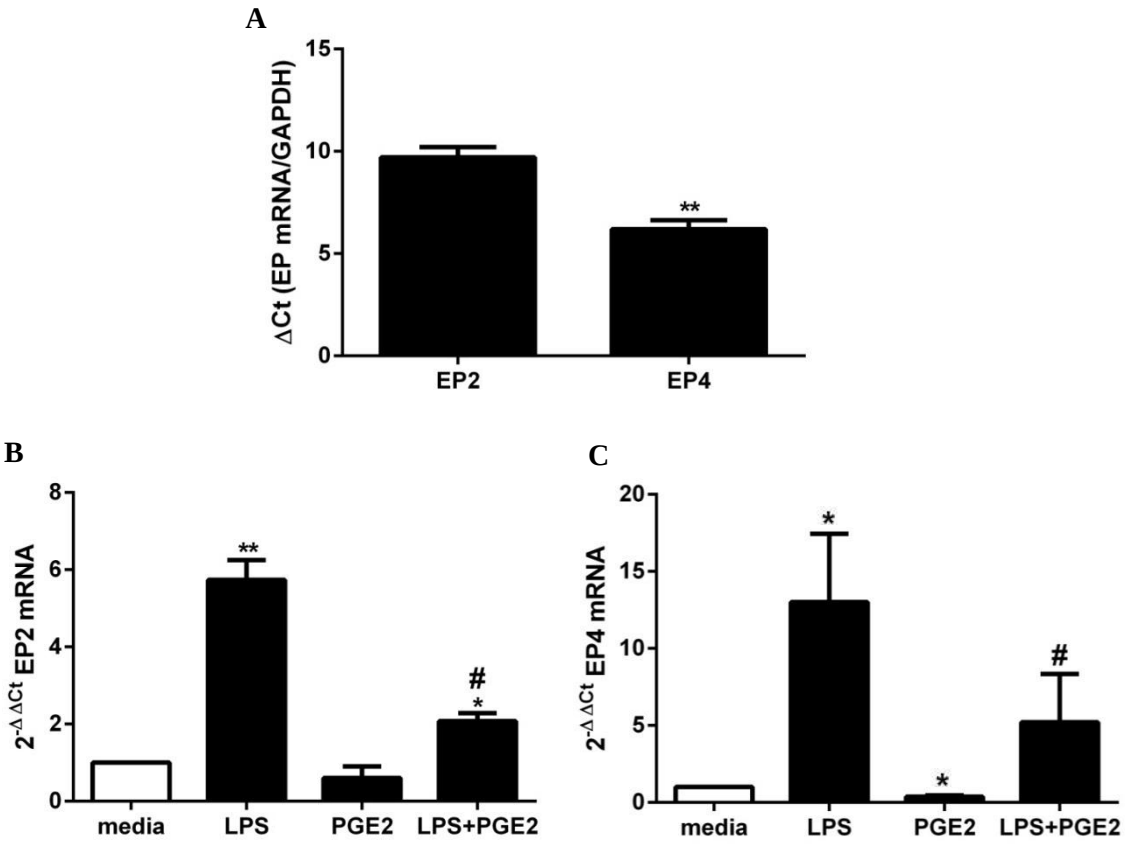


Figure 21: ***qPCR Confirms that LPS Induced PTGER2 and PTGER4 Expression is Attenuated by PGE2.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). mRNA was extracted from PMN directly following the PMN preparation using the TRI method (as described in chapter 2.3.). Total cellular RNA was reverse transcribed into cDNA using random primers. Resulting cDNA was then analysed by qPCR using primers for *PTGER2* (EP2; Panels **A**, **B**), *PTGER4* (EP4; Panels **A**, **C**) and *GAPDH* as a normalisation control. PMN were incubated for 4 hours with LPS (1 ng/ml), PGE2 (10 μM) or both. Basal expression of EP4 mRNA was greater than basal EP2 mRNA expression (Panel **A**). LPS upregulated EP2 and EP4 mRNA expression at 4 hours in PMN (Panels **B**, **C**). PGE2 alone reduced EP4 expression at 4 hours, and likewise reduced LPS-induced EP2 (Panel **B**) and EP4 (Panel **C**) mRNA expression levels by qPCR. Statistical analysis was performed through t-tests (Panel **A**) or One Way RM ANOVA with Dunnett's posttests (Panels **B**, **C**) on normalised Ct values (ΔCT), and displayed on graphs for $2^{-\Delta\Delta CT}$, where applicable. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. ***** denote differences to untreated conditions. **#** indicate differences to LPS treatment conditions. Results were considered to be statistically significant for $p<0.05$ (*/#) and $p<0.01$ (**/##).

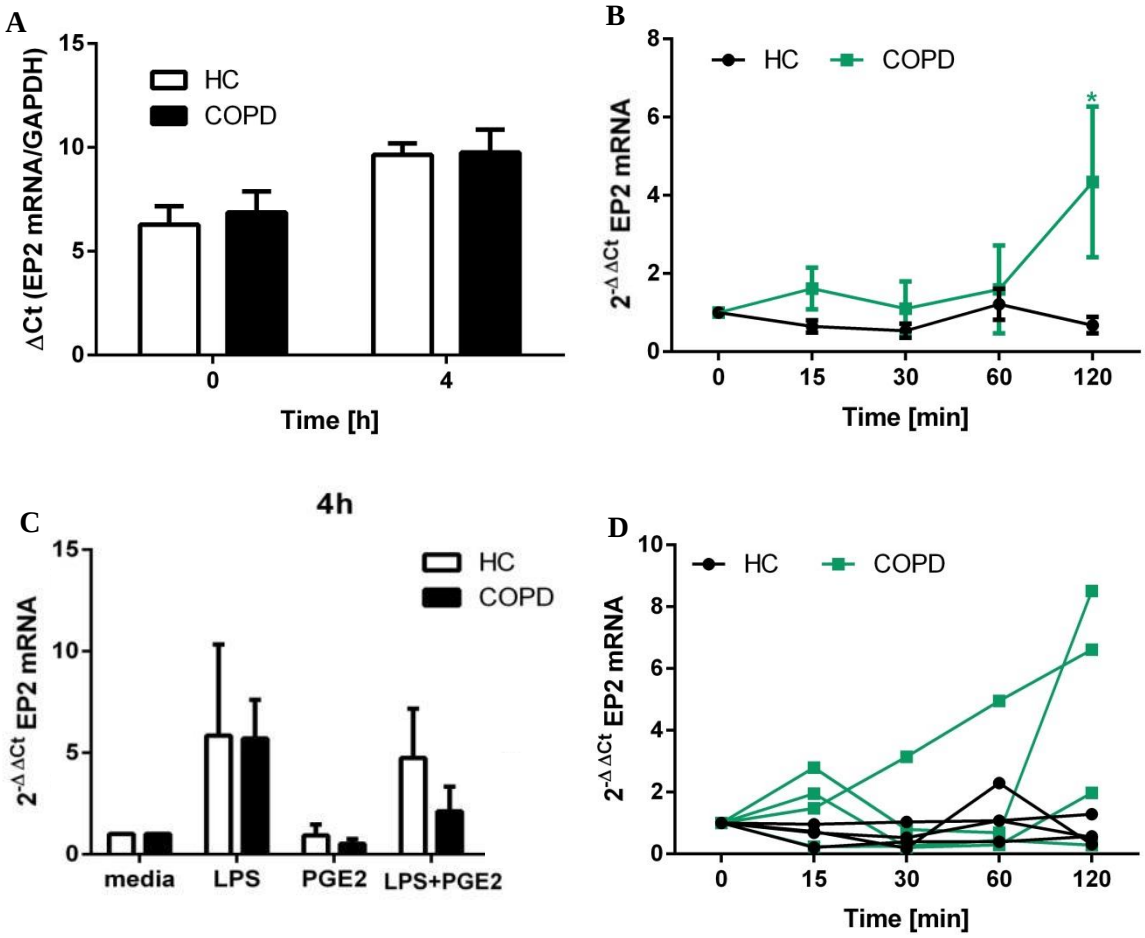


Figure 22: ***Enhanced PTGER2 Gene Transcription in LPS Primed PMN.*** Ultrapure PMN from healthy volunteers or COPD patients were incubated for 0 hours (Panel **A**), 0 - 2 hours with 10 ng/ml LPS (Panels **B, D**) or incubated for 4 hours (Panel **A**) with either 10 ng/ml LPS, 10 µM PGE2 or both (Panel **C**). mRNA was extracted from PMN using the TRI method (as described in chapter 2.3.). Total cellular RNA was reverse transcribed into cDNA using random primers. Resulting cDNA was then analysed by qPCR using primers for *PTGER2* and *GAPDH* as a normalisation control. EP2 mRNA expression for cells cultured in plain medium was not significantly different between healthy control and COPD patient PMN (Panel **A**). *PTGER2* expression was upregulated in COPD patient PMN at 2 hours in presence of 10 ng/ml LPS (Panels **B, D**). Individual data points for the assay in Panel **B** showed that 2 of 4 COPD patient PMN samples had upregulated EP2 mRNA expression at 120 minutes (Panel **D**).There were no statistically significant changes in *PTGER2* expression between healthy control and COPD patient PMN at 4 hours. Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttest on normalised Ct values (ΔCT), and displayed on graphs for $2^{-\Delta\Delta CT}$, where applicable. Data shown are mean ± SEM of 4 (Panels **B, D**) or 3 independent experiments (Panels **A**, **C**) with cells isolated from distinct volunteers, as described in chapter 2.5. ***** denote significant differences to untreated conditions and **#** indicate differences between corresponding treatments for healthy volunteers and COPD patients. Results were considered to be statistically significant for $p<0.05$ (*/#). Abbreviations: HC – healthy control.

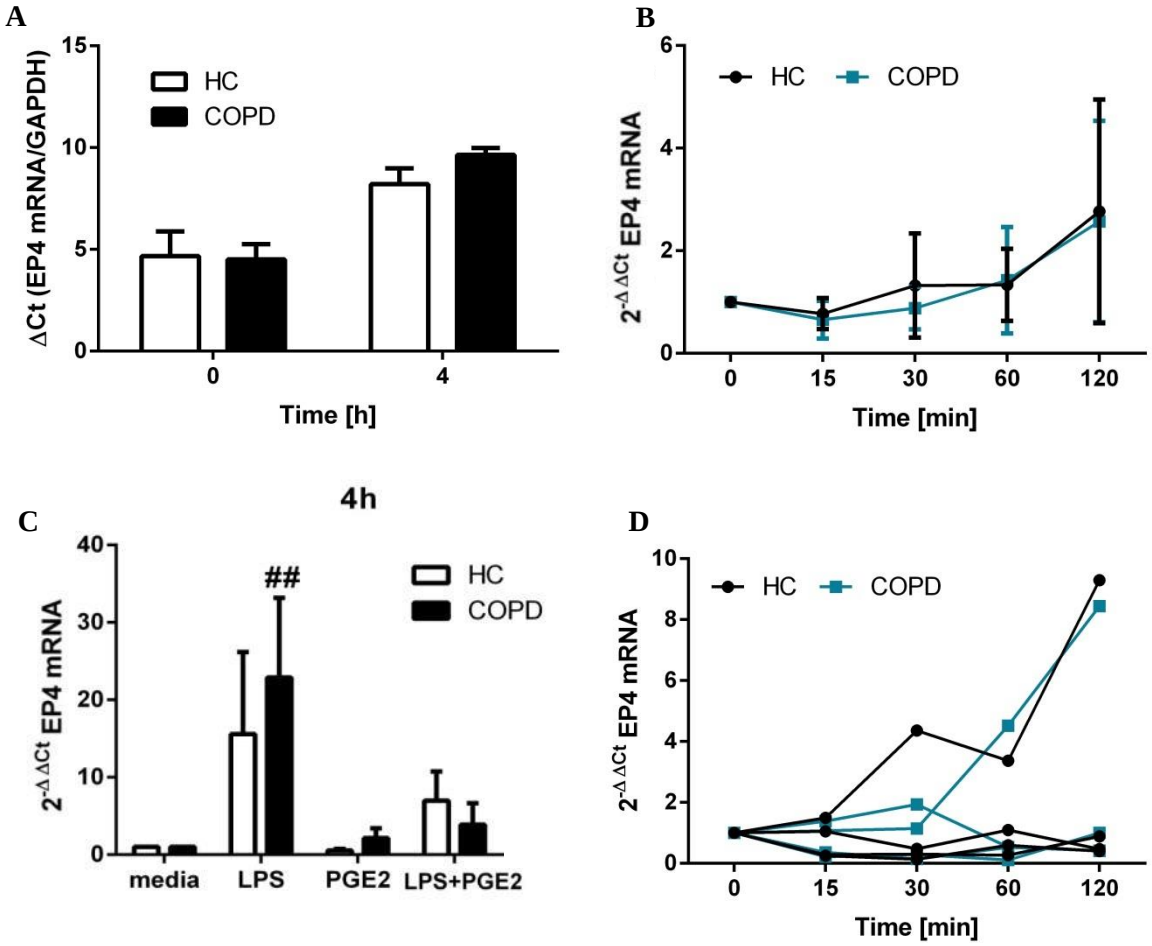


Figure 23: ***Enhanced PTGER4 Gene Transcription in LPS Primed PMN.*** Ultrapure PMN from healthy volunteers or COPD patients were incubated for 0 hours (Panel **A**), 0 - 2 hours with 10 ng/ml LPS (Panels **B, D**) or incubated for 4 hours (Panel **A**) with either 10 ng/ml LPS, 10 µM PGE2 or both (Panel **C**). mRNA was extracted from PMN using the TRI method (as described in chapter 2.3.). Total cellular RNA was reverse transcribed into cDNA using random primers. Resulting cDNA was then analysed by qPCR using primers for *PTGER4* and *GAPDH* as a normalisation control. EP4 mRNA expression for cells cultured in plain medium was not significantly different between healthy control and COPD patient PMN (Panel **A**). *PTGER*4 expression was non-significantly upregulated in healthy control and COPD patient PMN at 2 hours in presence of 10 ng/ml LPS (Panels **B, D**). Individual data points for the assay in Panel **B** showed that one PMN sample for each patient group had upregulated EP4 mRNA expression at 120 minutes (Panel **D**). Statistically significant differences in *PTGER4* expression were detected between healthy control and COPD patient PMN at 4 hours in presence of LPS (Panel **C**). Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttest on normalised Ct values (ΔCT), and displayed on graphs for $2^{-\Delta\Delta CT}$, where applicable. Data shown are mean ± SEM of 4 (Panels **A**) or 3-4 independent experiments (Panels **B, D**) with cells isolated from distinct volunteers, as described in chapter 2.5. ***** denote significant differences to untreated conditions and # indicate differences between corresponding treatments for healthy volunteers and COPD patients. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##). Abbreviations: HC – healthy control.

transient, as the difference between COPD and healthy control PMN was lost at 4 hours in a subsequent experiment (**Fig. 22C**). mRNA expression for EP4 was increased in healthy control PMN and significantly upregulated in COPD PMN (**Fig. 23C**). After 4 hours of LPS treatment, EP4 expression was significantly enhanced in COPD PMN. Treatment with PGE2 did not regulate EP2 or EP4 mRNA expression levels in COPD or healthy control PMN. However, in COPD PMN only, PGE2 blocked LPS-induced upregulation of EP2.

The functional relevance of increased prostaglandin receptor mRNA expression on extracellular PGE2 signalling depends on the translation of the receptor mRNA into protein. Therefore, Percoll pure PMN were treated with 1-10 ng/ml LPS for 4 and 20 hours. Whole cell lysates were made as described in chapter 2, and subjected to SDS PAGE. Membranes were incubated with primary antibodies for EP2 (1:200), EP4 (1:100), or β actin (1:10000) and chemiluminescent images were analysed by densitometry. At 4 hours, LPS treatment did not modulate EP2 and EP4 protein expression (**Fig. 24A, B**), whereas at 20 hours, EP2 and EP4 receptor protein was dose-dependently, but not significantly increased (**Fig. 24C, D**).

It was recently proposed that neutrophils from COPD patients may be primed due to the previous exposure to cigarette smoke (Koethe *et al.*, 2000). Likewise, the ability of LPS to induce a primed state in PMN, and thus to enhance the secondary responses to inflammatory reagents has long been recognised (Condliffe *et al.*, 1996; Condliffe *et al.*, 1998). Consequently, I investigated, whether pretreatment with LPS could enhance PGE2 induced survival in PMN. For this, Percoll pure PMN from healthy control volunteers were incubated in presence of 10 ng/ml LPS for 20 hours. Subsequently, LPS-primed and unprimed PMN were incubated in the presence and absence of 10 μM PGE2 for a further 4 hours. At 24 hours, 94.0 % (± 5.1 SEM) of untreated PMN were apoptotic, and unresponsive to treatment with PGE2 (**Fig. 25A**). LPS treatment significantly enhanced PMN survival in presence and absence of PGE2. Remarkably, there was a significant 12.9 % (± 3.6 SEM) fold change difference in LPS-primed PMN at 24 hours, between PMN incubated in presence or absence of PGE2 (**Fig. 25B, C**).

3.3. Discussion

This chapter discusses influences that may affect the efficacy PGE2 in PMN survival, the involvement of a specific receptor subtype on PMN survival and the interactions of LPS on PGE2 signalling.

3.3.1. Influences on the Efficacy of PGE2 on PMN Survival.

PGE2 displays pro-survival effects in PMN (**Fig. 8A**), with an increased potency in PMN from COPD patients (**Fig. 8B**). PGE2-induced survival increases dose-dependently, with a maximal biological response observed for 10 μM, which is 500-fold to 10000-fold in excess of its Ki

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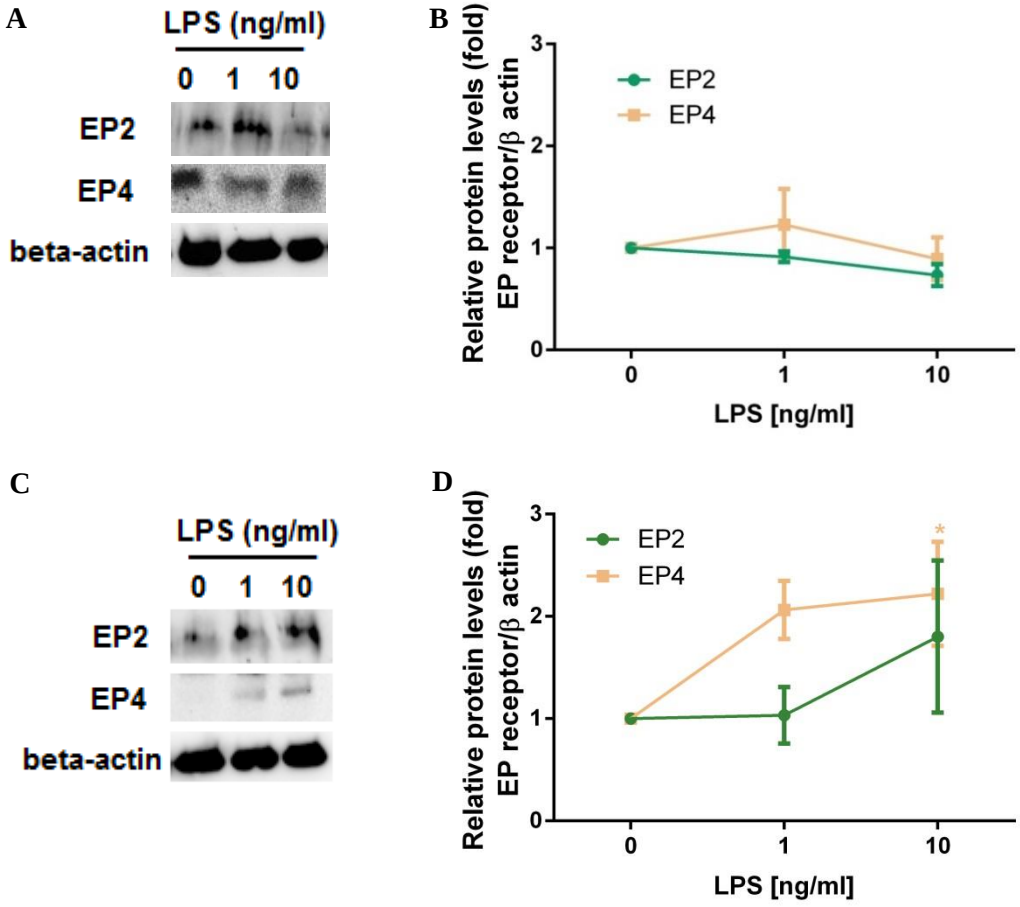
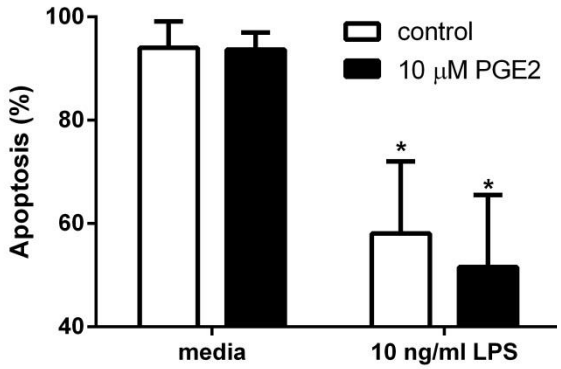
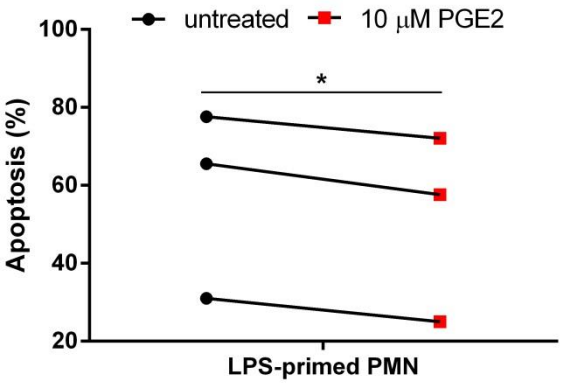


Figure 24: ***Modulation of Late EP2 and EP4 Protein Expression in Presence of LPS in PMN.*** Percoll pure PMN from healthy volunteers were incubated for 4 hours (Panels **A**, **B**) or 20 hours (Panels **C**, **D**) with LPS (1 - 10 ng/ml). Proteins were extracted from PMN with neutrophil lysis buffer (as described in chapter 2). Protein from 1 x 10^6 PMN/lane were loaded onto a 10 % SDS PAGE gel and transferred to nitrocellulose membranes, which were incubated with primary antibodies for EP2 (1:200), EP4 (1:100), or β actin (1:10000). Treatment with 10 ng/ml LPS non-significantly increased EP2 and EP4 protein expression at 20 hours in PMN (Panels **C**, **D**), but not at 4 hours (Panels **A**, **B**). Densitometry was undertaken using the ImageJ software. Statistical analysis was performed through two-tailed t-tests compared to the media control. Data shown are representative images (Panel **A**), or mean ± SEM (Panels **B**, **D**) of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Results were considered to be statistically significant for $p<0.05$ (*).

A



B



C

Samples (n=3)		Mean ± SEM	Mean difference ± SEM	t-test
LPS-primed PMN	untreated	58.1 ± 14.0	-12.9 ± 3.6	0.0130 (*)
	10 μM PGE2	51.6 ± 13.9		

Figure 25: ***LPS Pretreatment Enhances PGE2 Survival in PMN.*** Percoll pure PMN from healthy volunteers were pre-incubated for 20 hours with LPS (10 ng/ml). Subsequently, PMN were incubated in presence or absence of 10 μM PGE2 for a further 4 hours. PGE2 treatment alone did not significantly extend PMN survival at 24 hours (Panel **A**); however, PGE2 significantly downregulated apoptosis in LPS-primed PMN at the same timepoint (Panel **B**, **C**). Statistical analysis was performed through Two Way RM Way ANOVA with Sidak's posttests (Panel **A**), or paired (two-tailed) t-test (Panels **B**, **C**). Data shown are mean ± SEM of 3 independent experiments (Panels **A**, **B**) with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. ***** denote significant differences to control conditions. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##), $p<0.001$ (***/###) and $p<0.0001$ (****/####).

(0.85 – 20 nM) towards the receptors EP1, EP2, EP3 and EP4 (**Table 5**). It was postulated that the lower than expected efficacy of PGE2 was caused by a cytotoxic effect induced by the solvent dimethyl sulfoxide (DMSO). However, DMSO did not induce apoptosis in PMN at the concentrations used in this study (**Fig. 9**). Agonism of FP receptors increased PMN survival and FP receptor antagonism likewise blocked PGE2-induced survival to a low extent (**Fig. 10**), as did PPARγ antagonism (**Fig. 11**). The stable PGE2 analogue dmPGE2 induced PMN survival comparable to PGE2, but while PGE2 survival was lost at 20 hours, dmPGE2 sustained its effect on survival at the late time point (**Fig. 12**).

3.3.1.1. Influences of Solvent and Serum on PMN Apoptosis.

Several authors have recently described the pro-apoptotic effect of the common solvent DMSO (Hanslick *et al.*, 2009; Banic *et al.*, 2011; Galvao *et al.*, 2014) in the central nervous system of mice (Hanslick *et al.*, 2010) and through the interference with Casp-9-mediated apoptosis in hepatocytes (Banic *et al.*, 2011).

Here, only 1 % DMSO induced a low increase in PMN apoptosis (**Fig. 9**). As the highest concentration of DMSO used in the following assays of this study was lower than 0.1 %, it was therefore concluded that the effect of the solvent on PMN apoptosis is negligible. It is conventional to supplement culture medium with 10 % FCS. To reduce potential secondary variability induced by FCS, a single batch of heat-inactivated FCS (# C-37372) was used throughout this study. The heat-induced inactivation of trypsin was verified by the supplier, and the functionality of further enzymes was greatly reduced (informal communication).

However, the introduction of FCS in the cell culture medium encompasses the risk of allowing plasma protein to bind to PGE2 or its cell surface receptors. PGE2 binds readily to human serum albumin at 37 °C (Romanovsky *et al.*, 1999). Binding of plasma proteins has previously been proposed to explain a lack of efficiency of ligands at the receptor level (Coleman *et al.*, 1985c), which might lead to a reduced potency of PGE2 in reducing apoptosis. The normal level of albuminin blood is 3.4 – 5.4 g per decilitre, and with a high association constant of 2.4×10^4 (unitless) for PGE2 with human serum albumin (Raz, 1972), this might indicate that a considerable proportion of PGE2 could be bound in a physiological setting. Therefore, the addition of FCS might model *in vivo* conditions, as a lack of growth factors might otherwise induce the development of a withdrawal phenotype with increased levels of PMN apoptosis. Nevertheless, the effect on FCS binding free PGE2 in the culture medium is likely of only minor importance for this study. In support, the affinity of bovine serum albumin to human PGE2 is thought to be considerably decreased compared to its affinity for bovine PGE2 (Raz, 1972). In a serum free environment, PGE2 moreover significantly affected PMN function at 100 nM (Armstrong, 1995; Talpain *et al.*, 1995), which is only 10 fold lower than the concentration that significantly reduced PMN apoptosis in this study (1 μM). Future work to substantiate this

hypothesis may involve supplementing the PMN culture medium with varying concentrations of FCS, or BSA.

3.3.1.2. Pro-Survival Effects of PGE2 at Ultra-Ki Concentrations.

The need for relatively high (micromolar) PGE2 concentrations might also reflect a physiologically relevant effect. Biphasic and dose dependent actions of PGE2 signalling have been reported in various cell types (Coleman, Kennedy, 1980; Eglen, Whiting, 1988; Sergeeva *et al.*, 1997; Tang *et al.*, 2000; Zhang, Wang, 2014). In a study on the bronchoconstrictory effects of prostaglandins, the investigated prostaglandins were used at low micromolar concentrations, and the inefficacy of the PGs at lower concentrations was attributed to the involvement of the TP receptor, which had high Ki values for the prostaglandins in question (Coleman *et al.*, 1989). Moreover, biphasic effects of PGE2 in the guinea pig trachea, where only the initial proportion of the contractile curve was antagonised by an EP receptor antagonist, were attributed to the involvement of an additional receptor subtype (Eglen, Whiting, 1988). In this study, PGE2 induced a delayed dose-dependent survival in a potentially biphasic sigmoidal manner (i.e. **Fig. 10-12**), suggesting that PGE2-induced survival involves the action of additional receptor subtypes to the classical PGE2 receptors EP1, EP2, EP3 and EP4.

Therefore, a potential loss of specificity through the engagement of an additional receptor subtype for micromolar PGE2 concentrations was examined. With respect to the published receptor affinities of PGE2 (**Table 6**), the agonist has a Ki value of 100 nM for the FP receptor subtype. However, by using a FP receptor specific antagonist, only a modest inhibitory effect on PGE2 induced survival for 0.1 μM PGE2 was determined (**Fig. 10C**). This indicated that the engagement of the FP receptor subtype through PGE2 is possible and might influence the pro-survival effect of PGE2 in PMN. However, the effects of FP receptors on PGE2 induced survival are very limited, and at an antagonist concentration that matched the highest concentration of PGE2 used (10 μM), it is unlikely that PGE2 with its comparatively low affinity towards FP displaced AL 8810 from its receptor at concentrations below 10 μM. However, in some (**Fig. 10C**), but not all assays (**Fig. 10B**), AL 8810 increased constitutive PMN survival, which hinders the interpretation of the impact of FP receptors on PMN survival. This may be caused by variability in the basal protein expression of FP receptors.

3.3.1.3. Possible metabolism of PGE2 to PGF2α.

The dual effects of the prostaglandin PGD2 on inflammation have previously been reviewed (Sandig *et al.*, 2007). It was argued that PGD2 was rapidly metabolised and was able to exert further functions through its metabolic products. Interestingly, PGE2 shows similar dual roles in many cell types (Nishigaki *et al.*, 1996) and is also considered as metabolically unstable. Moreover, the prostaglandin receptors EP2 and EP4 have shown different sensitivity to metabolic degradation products of PGE2, with EP4 being more susceptible to desensitization

and PGE2 metabolism. Therefore, poor lipid stability has to be taken into account, when investigating PGE2 induced survival *in vitro*, while high local PGE2 concentrations might be able to sustain PMN survival responses *in vivo*, irrespective of PGE2 degradation.

It was therefore investigated, whether products of a potential protein-based catabolism of PGE2 may exert an influence on PMN lifespan, or were responsible for reducing the efficacy of PGE2 in inducing PMN survival. Intriguingly, the PGE2 metabolite PGF2α only decreased PMN apoptosis at 10 μM, to a similar extent as PGE2 at the same concentration (**Fig. 10A**). While these results corroborate the finding of Ward *et al.* (2002) for 10 μM PGF2α at a 4 hour timepoint, an additional, more complex role for PGF2α in the regulation of PMN survival was found. As micromolar concentrations of PGF2α were required to induce PMN survival, it is unlikely that PGE2 metabolism is responsible for the hypothetical engagement of the FP receptor.

An alternative hypothesis to a potential interference of PGE2 metabolism, PGF2α had a pro-apoptotic effect on PMN survival at low agonist concentrations. Therefore, it is possible that the non-specific engagement of the FP receptor at pharmacological concentrations of PGE2 masks the pro-survival effect of PGE2. However, PGE2 had a very low effect on PMN survival at sub-Ki concentrations for the FP receptor. Hence, the impact of this effect on PMN survival is likely to be minute.

3.3.1.4. Non-specific action through metabolism to 15-keto PGE2.

The 15-PGDH mediated degradation of PGE2 to its metabolite 15-keto PGE2 (**Fig. 3**; Polet, Levine, 1975; Hamberg, Samuelssen, 1971; Nishigaki *et al.*, 1996) may allow for inflammatory gene expression and side-effects on PMN survival induced by the targeting of PPARγ. Consistently, there is increasing evidence for a physiological role of PPARγ in inflammation (Rigamonti *et al.*, 2008). In fact, targeting of PPARγ has recently been proposed as a strategy in the regulation of neutrophilic inflammation in airway diseases, as PPARγ agonism blocked neutrophilia induced by inflammatory stimuli *in vivo* (Birrell *et al.*, 2004). In contrast to PGF2α, 15-keto PGE2 possesses a considerably attenuated affinity towards prostaglandin receptors, and is thought to have reduced physiological activity in comparison to PGE2 (**Table 5**; Nishigaki *et al.*, 1996; Hansen, 1976; Chi *et al.*, 1996), because of which the effect of its downstream effector PPARγ on PMN survival was tested here.

The PPARγ agonist rosiglitazone dose-dependently decreased PMN apoptosis for concentrations above 10 nM (**Fig 11A**), which might be consistent with its reported Ki of 6.92 nM for PPARγ receptors (Liu *et al.*, 2001). However, at sub-Ki concentrations (1-10 nM), rosiglitazone profoundly increased PMN apoptosis (**Fig. 11A**). This might indicate that a threshold level of receptor activation is required to show an effect for PPARγ agonism. However, it is also possible that rosiglitazone acts as an agonist on a second target at pre-Ki

concentrations. In fact, rosiglitazone also activates AMP-activated protein kinase (AMPK; Fryer, Parbu-Patel, Carling, 2002), which has been shown to be involved in the regulation of apoptosis. The increase in PMN apoptosis at low rosiglitazone concentrations was thus possibly mediated by a non-specific effect of the reagent only. Taken together, rosiglitazone did not significantly alter PMN survival at high reagent concentrations in respect to constitutive apoptosis. This is consistent with the findings of Ward *et al.* (2002), who were unable to alter apoptosis even with high PPARγ agonist concentrations.

To further define the role of PPARγ in PMN survival, the irreversible, selective PPARγ antagonist GW 9662 was employed. GW 9662 alone did not significantly increase PMN apoptosis, indicating that PPARγ is not constitutively expressed, or its signalling not constitutively active. However, GW 9662 blocked PGE2 induced survival at 100 nM agonist concentration, indicating that PPARγ might have a small pro-survival role in PGE2-induced survival. However, PGE2 survival at concentrations higher than 0.1 μM was not significantly decreased by GW 9662 treatment, suggesting that the antagonist might be displaced from the PPARγ receptor.

However, Lea *et al.* (2014) were unable to show the expression of PPARγ in human lung sputum neutrophils, questioning the relevance of the data obtained with the pharmaceuticals used to target PPARγ activity in this study. Nevertheless, PPARγ expression might be regulated through the induction of pro-survival gene expression, or in a temporal manner.

Therefore, a distinct approach was used to determine the specific involvement of the PPARγ receptor with the 15-PGDH inhibitor dmPGE2. The PGE2 analogue decreased apoptosis to a similar extent as PGE2 at 10 μM (**Fig. 12A**); however, its efficiency at the lowest concentration range tested was significantly reduced, resulting in a right shift in comparison to the PGE2 survival curve. It is well-known that the extent of dmPGE2 efficiency in comparison to PGE2 varies in distinct cell types (Coleman *et al.*, 1987; Eglen, Whiting, 1988), potentially dependent on the constitutive activity of PGDH metabolism in the corresponding cell type.

However, the lower efficiency of dmPGE2 at low concentrations may also be attributable to the differences in receptor affinities between dmPGE2 and PGE2. dmPGE2 agonizes EP2 and EP3 receptors at a comparable scale as PGE2 (**Table 5**), but possesses a lower affinity to EP4 and FP receptors, and does not target the EP1 receptor subtype. Judging by its lack of affinity towards the EP4 subtype at very low concentrations (Ki 43 nM); whereas PGE2 has a Ki of 1.9 nM towards EP4 receptors, it is likely that its low efficiency may be grounded in the involvement of the EP4 receptor subtype in PMN survival. This hypothesis was further examined in chapter 3.1.2.

Interestingly, dmPGE2 significantly sustained survival at early and late timepoints by roughly 10 % (**Fig. 12B**), whereas PGE2 had lost its efficiency at the 20 hours timepoint, suggesting that the temporally short lived effects of PGE2 on PMN survival are indeed dependent on protein

degradation by 15-PGDH. Likewise, the low difference between both agonists at 4 hours indicate that protein degradation induced by 15-PGDH, and thus the agonism of PPARγ, is unlikely to affect assays on PMN survival at this timepoint. The low efficiency of dmPGE2 at the sustenance of PMN survival is not surprising, not only as the constitutive activity of PGDH in the culture medium is unknown, but as their effect on survival might also be limited by the cellular uptake of PGE2 (Nomura *et al.*, 2004).

3.3.1.5. Uptake of PGE2 into the perinuclear space.

The physiological regulation of exogenous PGE2 levels is achieved by several mechanisms additional to PGE2 degradation. A prostaglandin transporter (PGT) mediating the cellular uptake of cell surface PGE2 has been reported (Kanai *et al.*, 1995). Thus, PGT is able to regulate PGE2 bioavailability and potentially increases PGE2 concentrations in the perinuclear space (Spencer *et al.*, 1998). PGE2 uptake would allow for the (partial) intracellular degradation of PGE2, accompanied with a loss of efficiency at the induction of PMN survival. This might help to explain the low efficiency of both PGE2 and dmPGE2 in PMN survival (**Fig. 12A**). PGE2 uptake, combined with degradation by 15-PGDH might therefore explain the efficiency of dmPGE2 at late timepoints only. As PGE2 uptake is thought to be a slow and energy-consuming process, this will be most relevant for late timepoints in PMN survival.

Decreased availability of PGE2 at the cell surface might thereby control PGE2 signalling. However, perinuclear localisation of functional EP receptors has also been reported (Bhattacharya *et al.*, 1999; Konger *et al.*, 2005), and will be discussed in more detail the next subchapters.

In conclusion, the influences of the solvent DMSO and the serum used in this study are thought to be negligible. The activity of the enzymes CBR and 15-PGDH is likely to be reduced, but residual activity may induce PGE2 protein degradation to PGF2α and 15-keto PGE2. PGF2α and FP receptor engagement had a dual role on PMN survival, with pro-apoptotic effects in the nanomolar concentration range. Therefore, the pro-apoptotic effect of the FP receptors at low agonist concentrations may mask PGE2 induced survival, resulting in a lower efficacy of the reagent on PMN survival. On the other hand, engagement of PPARγ may have pro-survival roles, but its effects may be more relevant for the sustenance of late prostaglandin signalling. Therefore, it was postulated that PGE2 mediates PMN survival in a potentially biphasic manner through the involvement of distinct PGE2 receptor subsets, which will be further discussed in the next subchapter.

3.3.2. EP2 Phenotype in the induction of PGE2 survival in PMN

A molecular mechanism for biphasic effects of PGE2 was proposed as early as 1979 by Kather & Simon in human fat cells, who demonstrated that 0.1 – 10 nM PGE2 inhibited AC-mediated

cAMP release, whereas the cAMP release drastically increased for concentrations of 10 nM – 1000 μM. This correlates well with the reported Ki values for low- and high-affinity prostaglandin receptors at different concentrations and their cellular functions. Therefore, the specific involvement of the distinct PGE2 receptor subtypes was examined, to determine whether the inhibitory effect on PGE2 survival might be caused by the engagement of a distinct PGE2 receptor subtype.

Constitutive expression of EP2 and EP4, but not EP1 or EP3 was found (**Fig. 13**), with predominant expression of the EP4 receptor subtype. Agonists of EP2, EP3 and EP4 induced significant reductions in PMN apoptosis (**Fig. 14**). Antagonism of EP2 (**Fig. 16**) and EP4 receptors (**Fig. 17**) blocked PGE2-induced survival, with a rank order of potency EP2>EP4. Similarly, antagonism of both EP2 and EP4 blocked PMN survival that was not significantly different from antagonism of EP2 alone (**Fig. 18**).

3.3.2.1. Basal Prostaglandin Receptor Expression in PMN.

RT-PCR analysis detected mRNA expression for EP2 and EP4 receptors (**Fig. 13A, B**). Consistently, basal expression of EP2 and EP4 mRNA was found in murine peritoneal neutrophils (Yamane *et al.*, 2000) and murine RAW 264.7 macrophages (Arakawa *et al.*, 1996; Hubbard *et al.*, 2001), J774.1 macrophages (Katsuyama *et al.*, 1998), and EP4 expression only in murine peritoneal macrophages (Ikegami *et al.*, 2001). Constitutive expression of EP4 mRNA was highest, compared to the expression of the other PGE2 receptors in PMN and a low level of EP2 expression was detected (**Fig. 13A, B**). This is consistent with the constitutive expression of EP2 and EP4 in RAW 264.7 macrophages (Hubbard *et al.*, 2001).

In this assay, expression for EP1 and EP3 was not detected, consistent with the lack of RT-PCR expression of EP1 and EP3 in murine peritoneal neutrophils (Yamane *et al.*, 2000). In contrast, low levels of EP3 were found in RAW 264.7 macrophages (Hubbard *et al.*, 2001). However, EP1 and EP3 receptors have been proposed to possess higher protein stability and lower receptor turnover rates. Therefore, EP1 and EP3 receptors might be present on the cell surface despite lacking mRNA expression. This is consistent with the findings of Yamane *et al.* (2000), who detected EP1 and EP3 on the protein level, but not expressed as mRNA. Consequently, the functional relevance of EP2 and EP4 mRNA expression was likewise confirmed by western blotting (**Fig. 24**).

3.3.2.2. Influence of Prostaglandin Receptor Agonism on PMN Survival.

Selective agonists for the PGE2 receptors EP1, EP2, EP3 and EP4 were used to determine which PGE2 receptor subtype mediated PGE2 effects on PMN survival. The concentration ranges of the selective agonists correspond to the range of PGE2 concentrations where significant differences on PMN survival were previously detected (**Fig. 7A**). Notably, all agonists possess comparable or lower affinity towards their respective receptors than PGE2

(**Table 6**). The EP1 agonist 17-phenyl trinor PGE2 induced only a very low level of apoptosis at a concentration of 1 μM (**Fig. 14A**), which correlates with its inability to specifically distinguish between the EP1/EP3 and EP4 receptor subtype, because of its gain in EP4 affinity (**Table 6**). This indicates that either EP1 receptor agonism possesses a lower efficacy in mediating PMN survival, or that it is not expressed by human PMN.

The EP2 agonist butaprost mirrored the PGE2 curve and was equally efficacious in prolonging PMN lifespan (**Fig. 14B**). However, butaprost possesses a more than 9 fold lower affinity towards the EP2 receptors than PGE2 (**Table 6**), indicating that the EP2 receptor subtype binding is involved in the transmission of PGE2 survival effects in PMN.

With the EP3 agonist sulprostone, the initial proportion of the dose-response curve was shifted to the left from the PGE2 curve (**Fig. 14C**). This shift might be in keeping with the 1.4 fold higher affinity of the agonist towards the EP3 receptor in comparison to PGE2 (**Table 6**). This suggests an equipotent influence of the agonist on PMN survival with PGE2. A steep increase in sulprostone induced PMN survival followed for agonist concentrations above 1 μM, suggesting the engagement of an additional receptor subtype by the agonist. In the last part of the curve, sulprostone loses its specificity and targets FP receptors at a Ki of 580 nM (**Table 6**), coinciding with the increased levels of apoptosis observed for the respective concentration.

The EP4 agonist L-902,688 was extremely potent and the tested concentration range was insufficient to capture its Ki (**Fig. 14D**). This was surprising, even when considering the approximately 7 fold higher affinity of the compound towards EP4 receptors when compared to PGE2. However, L-902,688 displayed a lower maximal efficacy than PGE2 at inducing PMN survival. Therefore, the agonist might have acted as a partial agonist on the receptor for concentrations below 1 μM, or its influences on PMN survival are limited. In conclusion, this finding warrants further investigation. In particular, because EP4 has been shown to be internalised and remain functional (Bhattacharya *et al.*, 1999). Thus, PGE2 might mediate further effects on survival signalling once it has become imported into the cell.

A steep increase in PMN apoptosis was observed for agonist concentrations of 3 μM to 10 μM for all agonists apart from the EP1 agonist. However, this is unlikely to be a non-specific effect of the solvent, as the solvent DMSO previously did not affect the extent of PMN lifespan in the concentrations used in this study (**Fig. 8**). This indicates that high agonist concentrations might induce cytotoxic effects, potentially through a loss of specificity towards their respective receptor subtype. As previously investigated, this might be mediated by the engagement of the FP receptor, as FP agonism was previously shown to induce apoptosis (**Fig. 10**).

3.3.2.3. EP2 and EP4 Receptor Antagonism Blocks PGE2 Induced Survival in PMN.

Due to the poor selectivity of the available receptor agonists, the use of selective receptor antagonists is commonly advisable in order to assign a functional role to individual receptors.

As previously elaborated, antagonism of EP3 in the context of PMN apoptosis has been previously examined elsewhere (Liu *et al*., 2005; Liu *et al*., 2007) and no functional role of EP3 in apoptosis was found. Furthermore, EP3 mRNA was not detected in this current study and therefore, antagonism of the EP3 receptor was not examined here.

The EP2 and EP4 selective antagonists PF-04418948 and GW 627368X were employed to confirm the preliminary findings of the agonist studies. Treatment of PMN with either antagonist alone, or a combination of both did not affect rates of constitutive PMN apoptosis (**Fig. 15**). However, PGE2 induced PMN survival was significantly delayed by both PF-04418948 and GW 627368X (**Fig. 16A, B**; **Fig. 17A, B**), as well as a combination of the antagonists (**Fig. 18A**), indicating that both receptors are involved in the mediation of pro-survival responses to PGE2. For both 300 nM and 1 μM PF-04418948, the PGE2 survival curve was shifted to a similar extent, overall inducing a higher level of apoptosis. However, at the highest concentration used, PF-04418948 was unable to block PGE2-induced survival, potentially suggesting that the agonist might displace the antagonist from the receptor, whereas the involvement of an additional PGE2 target at low micromolar concentrations is also a possibility.

The relative potencies of the agonists indicate that the EP2 receptor is the predominant receptor in PGE2 induced survival, in particular, when considering the higher constitutive expression of EP4 (**Fig. 13**). Consistently, butaprost-induced survival (3 μM) was significantly blocked by PF-04418948, suggesting that EP2 receptors are involved in the induction of PGE2 survival. Interestingly, treatment with 0.3 μM GW 627368X more prominently delayed PGE2 induced survival at an agonist concentration of 0.1 μM (**Fig. 17A**). This may suggest that the antagonist is displaced from the receptor by excessive PGE2 concentrations. However, a similar trend was observed with an increased GW 627368X concentration (1 μM; **Fig. 17B**). As the high-affinity receptor EP4 is preferentially targeted by low PGE2 concentrations, this infers that EP4 is involved in the transduction of the survival response at low PGE2 concentrations. Therefore, it is likely that high-affinity receptors mediate the actions of low PGE2 concentrations and vice versa. This corresponds well with the data in **Fig. 12A**, where dmPGE2 was less effective than PGE2 at low concentrations, and consistently has a significantly lower affinity towards EP4. Moreover, dmPGE2 previously displayed a lower efficiency at the induction of PMN survival than PGE2 (**Fig. 12**), which may be explained by its lower affinity towards EP4 receptors (**Table 5**). Consequently, EP2 may therefore predominantly induce PGE2 survival at high PGE2 concentrations.

A combination of PF-04418948 and GW 627368X was highly effective at blocking PGE2-induced survival, where only the highest PGE2 concentration (10 μM) was able to induce significant amounts of PMN survival (**Fig. 18A**). Nevertheless, 10 μM PGE2 was still significantly blocked by EP2 and EP4 antagonism. However, there was no significant difference

to PGE2-survival blocked with PF-04418948 alone, indicating that the activity of EP2 alone is sufficient to induce PGE2 survival. This suggests that EP2, and not EP4 is the main receptor in the transduction of early PGE2 survival in PMN, while both may show additive effects on PMN survival.

This suggests that even though both AC-coupled receptors EP2 and EP4 have previously been shown to increase intracellular cAMP, this highlights further non-redundant functions of the receptors in PMN survival. Interestingly, it was previously shown that PGE2-induced cAMP increases were not blocked by EP4 receptor antagonism (Armstrong, Talpain, 1994). However, the possibility was not excluded that a loss of EP2 might be partially compensated for by the EP4 receptor. Moreover, the detailed investigation of the involvement of the specific receptor subtypes involved in PGE2 survival is limited due to the insufficient specificity of the available pharmaceuticals. Antagonist specificity was compromised at concentrations that were required to prevent PGE2-induced competitive displacement of the receptor antagonists from their receptors. However, high PGE2 concentrations were needed to significantly influence PMN survival.

In particular, a further investigation of the role of the EP4 subtype is warranted, because it has previously been shown that the EP4, but not EP2 receptors lose their sensitivity upon ligand binding (Nishigaki *et al.* 1996) and become internalised (Bhattacharya *et al.*, 1999; Desai, Ashby, 2001). Functional EP3 and EP4 (Bhattacharya *et al.*, 1999), as well as functional EP1 and EP2 (Konger *et al.*, 2005) have been observed in the perinuclear space. Moreover, PGT-mediated PGE2 uptake (Kanai *et al.*, 1995) may regulate PGE2 signalling through decreased availability of PGE2 in the extracellular matrix (Chi *et al.*, 2014). This might represent an autoregulatory mechanism on PGE2 signalling through a negative feedback loop in PGE2 signalling. However, as PGT-mediated PGE2 uptake and Cox-2 initiated PGE2 expression may overall increase intracellular PGE2 levels under certain conditions, this may also indicate that functional perinuclear EP4 receptors (Bhattacharya *et al.*, 1999) might still be involved in the mediation of late PGE2-induced survival. Unfortunately, the permeability of the agonists and antagonists, and their potential interaction with intracellular (PGT-imported or LPS/Cox2 derived) PGE2 signalling, are unknown. Therefore, the influence of PGE2 and/or PMN activation in this scenario on the regulation of EP receptor expression was examined.

3.3.3. LPS induces EP2 and EP4 receptor expression and enhances late PGE2 survival.

In COPD, the presence of the pro-survival mediator PGE2 is increased (Ottonello *et al.*, 1998; Montuschi *et al.*, 2003; Dick *et al.*, 2009; Profita *et al.*, 2010), as is PMN survival (Perng *et al.*, 2004). LPS is a well-known inducer of COPD (Kobayashi *et al.*, 2013; Korsgren *et al.*, 2012), and it was thus hypothesized that both may cooperate in the induction of PMN survival.

EP1 and EP3 receptor mRNA expression could not be induced by treatment with LPS or PGE2 (**Fig. 19**). LPS non-significantly induced the expression of both EP2 and EP4, which was reduced by PGE2 in a RT-PCR (**Fig. 20**). This finding was confirmed by qPCR, where LPS induced expression of both receptors, blocked by PGE2 treatment (**Fig. 21**). In PMN from COPD patients, the expression of EP2 was already increased after 2 hours of LPS treatment (**Fig. 22**). Comparable amounts of LPS-induced EP4 expression were observed (**Fig. 23B**); however, EP4 expression was non-significantly upregulated in one PMN sample for healthy controls, as well as COPD patients, potentially indicating that upregulation of EP expression is not a defining feature of COPD. On the contrary, it may be a typical host-response to previous exposure to infections that might lead to upregulation of TLR receptor expression. EP4 expression was significantly increased by LPS in COPD PMN at 4 hours (**Fig. 23C**). mRNA expression was translated into protein by 20 hours, but not 4 hours of LPS treatment (**Fig. 24**). Late PMN-survival, sustained by LPS was furthermore enhanced by treatment with PGE2 (**Fig. 25**).

3.3.3.1. Regulation of Prostaglandin Receptor Expression by PGE2 and TLR4 Signalling.

Prostaglandin receptor expression was investigated as described (chapter 3.2.1.). Treatment with PGE2 did not significantly regulate EP1, EP2, EP3 and EP4 receptor expression (**Fig. 19**, **Fig. 20**) by RT-PCR. Interestingly, incubation with PGE2 significantly decreased EP4, but not EP2 mRNA expression by qPCR, consistent with the data by Ikegami *et al.* (2001), who proposed that EP4 expression is regulated by PGE2 through a negative feedback loop. Similarly, PGE2-mediated EP2 and EP4 downregulation was observed by Nishigaki *et al.* (1996) and Hubbard *et al.* (2001). Both Hubbard *et al.* (2001) and Ikegami *et al.* (2001) found that downregulation of EP mRNA was mediated through the activity of PKA in macrophages.

Here, LPS induced the expression of both EP2 and EP4 mRNA (**Fig. 21B, C**) and was attenuated by PGE2 treatment. This is consistent with Arakawa *et al.* (1996) and Pavlovic *et al.* (2006), who showed EP2 and EP4 upregulation upon LPS-stimulation in macrophages. LPS induced marked EP2 expression, and low levels of EP4 expression in J774.1 (Katsuyama *et al.*, 1998). EP2 upregulation in RAW 264.7 macrophages was thought to be mediated through the engagement of the p38/MAPK pathway (Hubbard *et al.*, 2001). Conversely, EP2 was potently induced by LPS treatment, whereas EP4 mRNA was downregulated in murine peritoneal macrophages (Ikegami *et al.*, 2001). LPS-induced suppression of EP4 expression was mimicked by PKA agonism, thus the LPS-induced decrease in EP4 mRNA was attributed to the action of LPS-induced PGE2 expression, which acted as a negative feedback loop (Ikegami *et al.*, 2001). This may be consistent with the findings from this study, as macrophages are thought to be the main source of LPS-derived PGE2 (Hubbard *et al.*, 2001). Therefore, the lacking presence of LPS-induced PGE2 may fail to control prostaglandin expression levels in PMN. Thus, these

findings suggest that a functional link between PGE2 and TLR4 signalling exists in the regulation of PGE2 induced PMN survival, potentially serving to prime PMN survival and/or inflammatory gene expression upon encounter of bacterial LPS.

3.3.3.2. Regulation of Prostaglandin Receptor Expression by TLR4 Signalling in COPD PMN.

It was investigated, whether LPS treatment can enhance PGE2 induced survival in the context of COPD through alterations in PGE2 receptor expression. LPS significantly enhanced EP2 mRNA expression at 2 hours in COPD PMN (**Fig. 22B**), where two out of 4 donor samples displayed a response (**Fig. 22D**). This may be consistent with the data by Ikegami *et al.* (2001), where EP2 mRNA was transiently upregulated by LPS, with a maximum biological response at 3 hours in macrophages. At 4 hours, COPD PMN were slightly more susceptible to PGE2 induced blocking of LPS-elicited EP2 upregulation (**Fig. 22C**), potentially through the early upregulation of EP2 receptor expression at 2 hours. This may be consistent with the increased IL-6 production through the PGE2/EP2 axis (Yamane *et al.*, 2000), and the highly increased levels of IL-6 in COPD PMN (Bucchioni *et al.*, 2003). All assays were performed with matched COPD and healthy control samples. However, the samples were not age matched, so it may be possible that the age of the participants may further contribute to the variability of the data. Interestingly, alterations in PKA signalling were previously observed for physiological cues, such as nutritional status and age (Pérez-Sieira *et al.*, 2014).

Interestingly, EP4 expression was not significantly increased in both COPD PMN and healthy control PMN at 2 hours (**Fig. 23B**). Consistently, PGE2 had a lower effect on LPS-induced EP4 expression at 4 hours (**Fig. 23C**). EP4 expression was strongly increased by LPS in COPD PMN, correlating with the strongly increased levels of TNFα in acute exacerbations in COPD (Daldegan *et al.*, 2005). This potentially suggests that COPD patient PMN may be more prone to display increased levels of PGE2 induced survival and a difference in the responsiveness to the induction of survival by PGE2 induced survival in LPS primed PMN may exist. This hypothesis was tested hereafter.

3.3.3.3. Expression Changes in EP2 and EP4 Receptor mRNA are Translated into Protein Expression.

A potential effect of mRNA upregulation onto functional outcomes for the cell firstly depend on the translation of prostaglandin receptor mRNA to protein. EP2 and EP4 protein expression in response to LPS was determined by western blotting at 4 hours and 20 hours as described (chapter 3.2.2.). At 4 hours, no significant changes in protein expression were observed (**Fig. 24A, B**). In contrast, the expression of EP2 and EP4 was increased at 20 hours, whereby only treatment with 10 ng/ml LPS significantly enhanced EP4 levels (**Fig. 24C, D**). This suggests that mRNA for EP2 and EP4 may potentially be translated into protein. However, if a

higher increase in EP receptor translation takes place, it may be observed at an intermediate timepoint and a further detailed investigation of the temporal regulation of EP2 and EP4 protein translation in healthy control and COPD PMN would be required to further substantiate this claim. Taken together, there is insufficient evidence to support a functional role of LPS in the regulation of the PGE2 survival response through EP receptor upregulation.

3.3.3.4. Functional Consequences of LPS-mediated EP2 and EP4 Receptor Upregulation.

To determine the functional relevance of EP receptor upregulation on PMN survival, PMN were pretreated with or without LPS for 20 hours, and then exposed to PGE2 for 4 hours. As previously observed (**Fig. 12B**), PGE2 had lost its effect on PMN survival at 20 hours (**Fig. 25A**). As EP2 and EP4 receptor expression was observed by western blotting, this effect may be linked to EP receptor desensitisation, and/ or internalisation. However, as untreated PMN are highly apoptotic at 20 hours, the general responsiveness to PKA activation may be reduced. Interestingly, pretreatment with LPS for 20 hours increased general PMN survival at 24 hours, and significantly increased the response to late PGE2 treatment (**Fig. 25B, C**). This effect may be a functional consequence of LPS-enhanced EP receptor expression (**Fig. 24C, D**), and may thus be even greater in COPD PMN, potentially contributing to the neutrophilia observed in the condition. However, the possibility cannot be excluded that the overall reduced levels of apoptosis in LPS-treated PMN contributes to their increased responsiveness to PGE2, but a further investigation may be required to further substantiate this hypothesis. Interestingly, Ikegami *et al.* (2001) proposed a functional switch from EP4 to EP2 in macrophage activation, which may induce differential cytokine expression, and exert an altered effect on functional responses. As this chapter showed a greater influence of EP2 on PMN survival, this may be consistent with Birrell *et al.* (2015), who conversely observed highly increased neutrophil numbers in EP4 KO mice in response to treatment with LPS or cigarette smoke. However not on a constitutive level, suggesting that the negative feedback loop exerted by PGE2 signalling is required for the maintenance of appropriate cell numbers in inflammation. Moreover, it was observed that PGE2 mediated its anti-inflammatory effects in the lung through the activation of the EP4 receptor subtype. Moreover, EP2 KO mice decreased neutrophilia. This is in contrast to Ikegami *et al.* (2001), who proposed a functional switch from EP4 to EP2 in macrophage activation, whereas EP4 was thought to be dominant in non-activated cells.

A more detailed investigation of the events involved in LPS-induced survival are required to shed light on the potential functional relevance of LPS priming in PMN on PGE2 induced PMN survival. Therefore, it was concluded that LPS may interact with PGE2 signalling events in PMN by enhancing PGE2 pro-survival receptor expression. However, a further investigation of the exact mechanisms of the potential functional relevance of this finding is warranted.

3.4. Summary

It was determined here that PGE2 exerts a possibly biphasic pro-survival effect in PMN, with a higher influence at low micromolar concentrations. The high amounts of PGE2 required to delay apoptosis were not affected by the influence of DMSO, or protein degradation at 4 hours. A potential non-specific targeting of PGE2 at FP receptors was observed and this might mask the pro-survival effect of PGE2 in PMN. Moreover, it was concluded that protein binding by serum might diminish the amount of free, extracellular PGE2 concentrations.

The engagement of the EP2 and EP4 receptor subtypes might both contribute to the pro-survival effect of PGE2 in PMN, as both are coupled to the stimulation of adenylyl cyclase (AC) and thus cAMP production (Sugimoto, Narumiya, 2007). However, the extent of EP2-induced survival was by far greater, which supports an EP2 phenotype in the extension of PMN survival.

EP2 and EP4 receptor expression was induced by LPS treatment, and downregulated through PGE2, indicating that a negative feedback loop may regulate PMN survival through LPS-induced PGE2 expression. Therefore, these findings propose a mechanism to the finding of the Sabroe/Whyte research groups, who observed that LPS-stimulation of neutrophils only transiently increased their survival via TLR4 and NF-κB activation. However, factors released by monocytes further prolonged neutrophil survival after the neutrophil TLR4 receptors lost responsiveness to LPS stimulation (Sabroe *et al.*, 2002a), which was attributed to factors secreted by macrophages. Here, it is proposed that LPS modulates prostaglandin receptor expression in PMN, and may PKA-dependently extend PMN survival through enhanced Cox2-derived PGE2 secretion in macrophages.

Moreover, this is the first study to show that COPD PMN are hyperresponsive to LPS-induced EP2 mRNA expression at 2 hours, and to EP4 mRNA expression at 4 hours in PMN. EP2 and EP4 protein expression was induced by a low extent at a late timepoint by LPS, and this coincided with increased responsiveness to PGE2 induced survival in PMN.

CHAPTER 4. PKA IS A PRINCIPAL MEDIATOR OF PGE2 INDUCED PMN SURVIVAL.

PKA signalling is invariably linked to PMN survival. The first comprehensive study to show the importance of raising cAMP to prolong PMN survival was by Rossi *et al.* (1995), where the cAMP analogue dbcAMP and the adenylate cyclase inhibitor forskolin potently inhibited PMN apoptosis at a late timepoint. Ottonello *et al.* (1998) added to this by demonstrating the PKA-dependence of the cAMP elevating agent PGE2 and likewise of an inhibitor of cAMP degradation (i.e. PDE4).

In inflammation, neutrophil survival can be prolonged through the exposure to cAMP-elevating agents, such as extracellular ATP, acting through P2Y11 receptors (Vaughan *et al.*, 2007). Inhibition of PKA-mediated survival of PMN in inflammation is thought to be an attractive strategy to achieve resolution of inflammation. PKA signalling is involved in many cellular functions and signalling pathways, including the regulation of lipid metabolism, epinephrine, glucagon, dopamine, and adrenergic signalling. However, the mechanisms involved in the transduction of the cAMP signal in PMN survival are less well characterised. In chapter 3, the particular involvement of the EP2 receptor subtype in PMN survival was found. EP2 is thought to increase intracellular cAMP levels, which is a strong activator of PKA in PMN, leading to the inhibition of apoptosis (Krakstad et al 2004). Hence, this chapter aims to elucidate the molecular mechanism of PGE2 mediated PMN survival and to investigate the role of the putative PKA secondary effectors nuclear receptor subfamily 4, group A, members 2 and 3 (NR4A2, NR4A3) to understand the molecular mechanisms in PGE2-induced survival in PMN, which may potentially contribute to the development of novel therapeutic strategies to combat neutrophilia in inflammatory diseases, such as COPD.

4.1. Limitations and Opportunities in the Exploration of PKA Signalling.

The investigation of PGE2-mediated survival signalling is hindered due to several reasons. In particular, the inability to differentiate between non-specific toxicity and targeted effects on survival signalling, the lack of specificity of pharmaceutical modulators at increasing reagent concentrations, influences of residual cell impurities in the cell preparation, and potential autocrine signalling that alters constitutive survival, are all able to mask the effect of specific inflammatory mediators with effects accumulating in the same readout.

Here, it was aimed to substantiate the central role of PKA in cAMP mediated survival by using selective agonists and antagonists and to determine the paracrine and autocrine influences on PMN survival.

4.1.1. PKA: Constitutive PMN Survival is Mediated by PKA.

Physiological stimulation of PKA is mainly achieved through the regulation of the second messenger cAMP, although different strategies can be employed to raise intracellular cAMP for PKA activation *in vitro* (**Fig. 26**). Firstly, this can be achieved by activation of adenylyl cyclase (AC), which synthesises cAMP from ATP (Taussig, Gillman, 1995; Scott, Mercer, 1995) and thus relies on the presence of intracellular ATP stocks. Secondly, cAMP levels can be raised by inhibiting its metabolism to 5' AMP, mediated by Phosphodiesterases (PDE; Scott, Mercer, 1995). However, since the discovery of the exchange protein directly activated by cAMP (Epac; Rooij *et al.*, 1998), reagents that mediate non-specific elevations of cAMP can no longer be regarded as specific PKA activators. Thus, in addition to exploring the effect of constitutive PDE4 inhibition by using the PDE4 inhibitor rolipram, the PKA selective agonist dbcAMP, and the site-selective PKA agonists N6-MB-cAMP and 8-AHA-cAMP were used to explore PKA-dependent survival in PMN.

4.1.1.1. Influence of PDE4 Inhibition on PMN Survival.

To evaluate the effect of constitutive PDE4 inhibition, ultrapure PMN were incubated with 0.001 – 10 μM of the PDE4 inhibitor rolipram. Rolipram did not significantly alter PMN survival at any of the concentrations tested (**Fig. 27**). However, at the two highest concentrations tested, there was a modest, non-significant decrease in PMN apoptosis.

4.1.1.2. Influence of PKA Agonism on PMN Survival.

cAMP analogues are often employed in scientific research to mimic physiological cAMP responses in cAMP responsive cell lines and probe for the relevance of PKA signalling for the functional output; here: PMN survival. PMN were incubated with 50 μM and/or 100 μM of the well-established, membrane-permeable cAMP analogue dbcAMP (**Fig. 28A**) for 4 and 20 hours. dbcAMP significantly delayed PMN apoptosis at both timepoints (**Fig. 28B, C**). However, the highest level of PMN apoptosis inhibition achieved with 100 μM dbcAMP was merely 7.6 % (± 7.4 SEM) at 4 hours and 13.1 % (± 5.6 SEM) at 20 hours. To account for secondary effects induced by the degradation of dbcAMP to BTA through esterases in the cell culture serum (**Fig. 26**), the use of metabolically stable PKA agonists is required to substantiate the effect of cAMP-mediated PMN survival.

The site-selective PKA agonist pair N6-MB-cAMP (**Fig. 29A**) and 8-AHA-cAMP (**Fig. 29B**) with affinity for site A (PKAR1A) and site B (PRKAR1B) of PKA type I (**Table 7**), respectively prolonged PMN survival in a dose and time dependent manner, reaching statistical significance at 6 hours (50 μM; **Fig. 29C, D**). In comparison to dbcAMP, treatment with 100 μM N6/8-AHA reduced PMN apoptosis by 22.2 % (± 3.1 SEM) at 6 hours and 36.7 % (± 3.3 SEM) at 20 hours.

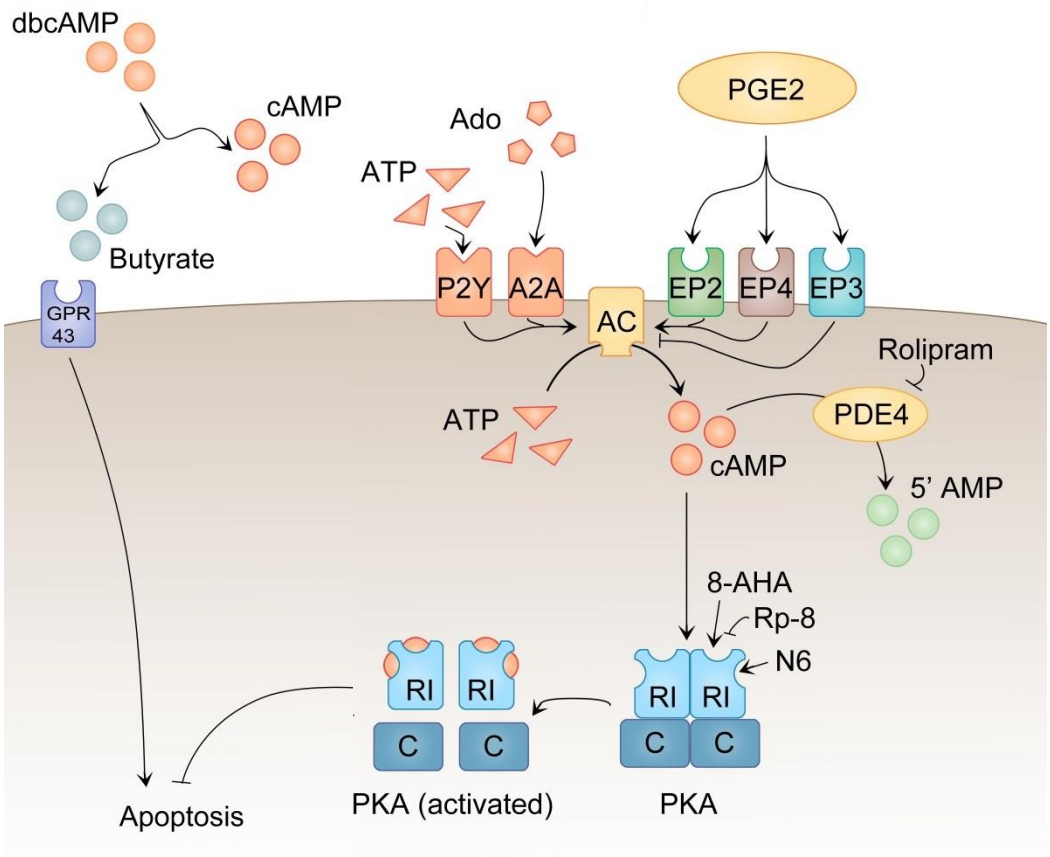


Figure 26: ***Diversity in the Activation of PKA Signalling.*** The release of the activated catalytic subunits from the PKA holoenzyme is achieved by the binding of two cAMP molecules to the A and B site of both regulatory subunits. Intracellualr cAMP stocks are replenished by the adenylyl cyclase (AC)-mediated conversion of ATP to cAMP. cAMP is degraded to the inactive compound 5' AMP by the Rolipram-inhibitable Phosphodiesterase 4 (PDE4). The activity of AC can be engaged by the activation of various receptors: the ATP-receptor P2Y11, the Adenosine-receptor A2A, the PGE2 receptors EP2 and EP4. dbcAMP can be degraded in the culture medium to the side-product butyrate and cAMP, which can be imported into the cell. Butyrate is implicated in apoptosis signalling and neutrophil activation through the action of GPR43. Abbreviations: C – PKA catalytic subunit; RI – PKA regulatory subunit I; 8-AHA – 8-AHA-cAMP; Rp-8 – Rp-8-Br-cAMPS; N6 – N6-AHA-cAMP; AC – adenylyl cyclase; Ado – Adenosine; P2Y – P2Y11 receptor; GPR43 – G-protein coupled receptor 43.

Table 7: Affinity (K_i) of PKA Agonists and Antagonists for PKA Subunits.

Ligands	Regulatory Type I		Regulatory Type II		References
	A (PRKAR1A)	B (PRKAR1B)	A (PRKAR2A)	B (PRKAR2B)	
cAMP	High (Ki N/K)	High (Ki N/K)	Low (Ki N/K)	Low (Ki N/K)	Pink, Dell'Acqua, 2003
N6-MB-cAMP (relative to cAMP)	3.60	0.093	0.74	0.041	Christensen *et al.*, 2003
8-AHA-cAMP (relative to cAMP)	0.055	4.1	0.010	0.39	Christensen *et al.*, 2003
8-Br-cAMP	>10000	N/K	>10000	426 ± 63	Griffin *et al.*, 1999
Rp-cAMPS	0.0013	0.0028	0.0021	0.022	Christensen *et al.*, 2003
Rp-8-Br-cAMPS	0.0008	0.00071	0.00048	0.094	Christensen *et al.*, 2003

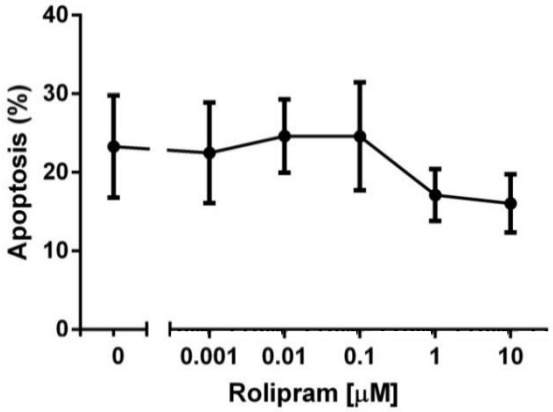


Figure 27: ***PDE4 inhibitor Rolipram does not Significantly Affect PMN Survival.*** PMN were separated from whole blood through Percoll density gradient centrifugation and negative magnetic selection. Following isolation, PMN were cultured at 1.67×10^6/ml in RPMI and 10% FCS with increasing concentrations of the PDE4 inhibitor rolipram (0.001 – 10 μM). Rolipram did not significantly influence PMN apoptosis at 4 hours. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Statistical analysis was performed through One Way RM ANOVA with Sidak's posttest. Results were considered to be not statistically significant for $p>0.05$.

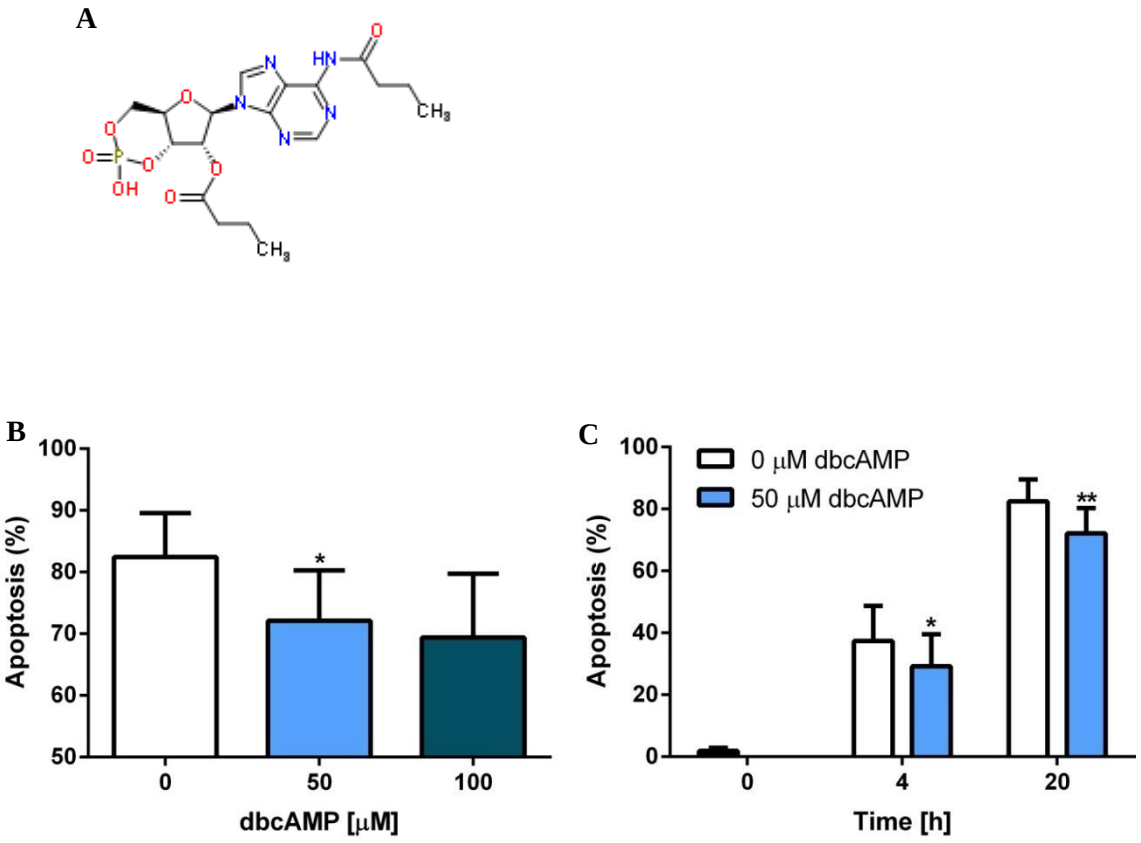


Figure 28: ***cAMP Analogue dbcAMP Prolongs PMN Survival Concentration and Time Dependently.*** PMN were separated from whole blood through Percoll density gradient centrifugation and negative magnetic selection. Following isolation, PMN were cultured at 1.67 × 10^6/ml in RPMI and 10% FCS. White bars are control conditions, while blue bars indicate incubation in the presence of 50 μM of the cell permeable cAMP analogue dbcAMP. Apoptosis was quantified by light microscopy of Wright/Giemsa stained cells. dbcAMP (Panel **A**) significantly delayed PMN survival in a dose dependent manner at 20 hours post-purification (Panel **B**) and time dependently with 50 μM dbcAMP (Panel **C**). Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Statistical analysis was performed through One Way RM ANOVA with Sidak's posttest (Panel **B**) or Two Way RM ANOVA with Sidak's posttest (Panel **C**). Asterisks (*) indicate differences to control conditions. Results were considered to be statistically significant for $p<0.05$ (*) and $p<0.01$ (**).

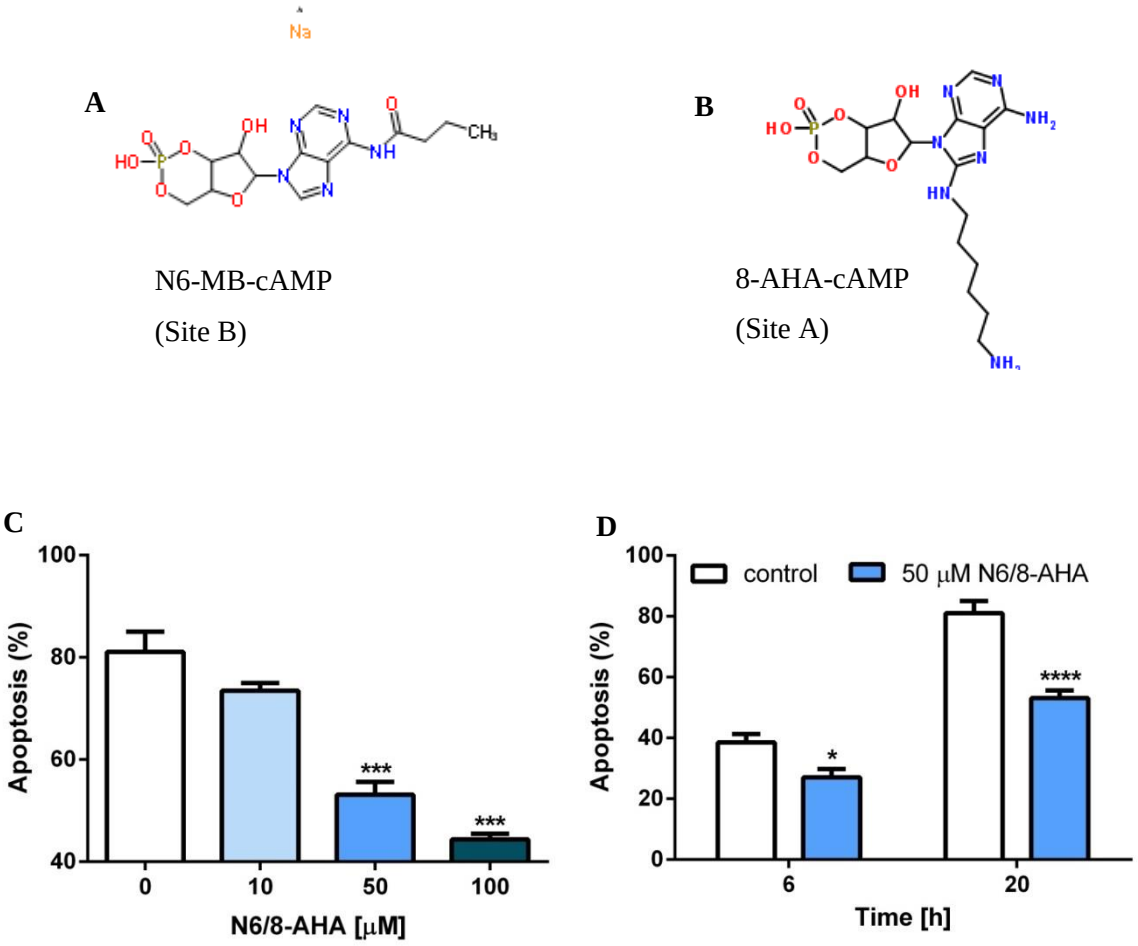


Figure 29: ***Selective PKA Type I Activation Enhances PMN Survival in a Concentration and Time Dependent Manner.*** PMN were separated from whole blood through Percoll density gradient centrifugation. Following isolation, PMN were cultured at 1.67×10^6/ml in RPMI and 10% FCS. White bars are control conditions, while blue bars indicate incubation in the presence of 50 µM or 100 µM of the PKA Type I site selective agonist pair N6-MB-cAMP and 8-AHA-cAMP. Apoptosis was quantified by light microscopy of Wright/Giemsa stained cells. cAMP analogues N6-MB-cAMP (Panel **A**) and 8-AHA-cAMP (Panel **B**) significantly delayed PMN survival in a dose dependent manner at 20 hours post-purification (Panel **C**) and time dependently with 50 µM N6/8-AHA (Panel **D**). Data shown are mean ± SEM of 4 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Statistical analysis was performed through One Way ANOVA with Dunnet's posttest (Panel **C**) or Two Way RM ANOVA with Bonferroni's posttest (Panel **D**). Asterisks (*) indicate differences to control conditions. Results were considered to be statistically significant for $p<0.05$ (*), $p<0.001$ (***) and $p<0.0001$ (****).

4.1.1.3. Influence of PKA Antagonism on PMN Survival.

The PKA inhibitor Rp-8-Br-cAMPS (**Fig. 30A**) prevents the activation of the enzyme by blocking cAMP binding sites. Highly pure PMN were incubated for 4 hours in the presence or absence of Rp-8-Br-cAMPS. It was found here that 0.7 mM Rp-8-Br-cAMPS increased constitutive PMN apoptosis by 12.8 % (± 2.7 SEM.) at 4 hours (**Fig. 30B**). The efficacy of Rp-8-Br-cAMPS was significantly greater in a proportion of assays, with a cut-off line at > 10 % mean difference between apoptosis in presence and absence of Rp-8-Br-cAMPS. In half of the assays, Rp-8-Br-cAMPS had a low efficacy and induced a mean percentage apoptosis increase of only 4.6 % apoptosis (± 1.2 SEM). In contrast, Rp-8-Br-cAMPS increased apoptosis with a high efficacy by a mean of 22.2 % (± 2.6 SEM; **Fig. 30C**) in the other moiety. This difference was not correlated with the level of constitutive apoptosis in PMN or the origin of the donation (data not shown). In summary, these data indicate that a low constitutive level of PKA signalling may be active in PMN.

4.1.2. Paracrine and Autocrine Regulation of PMN Survival.

Previously, low levels of PBMC were shown to prolong PMN survival (Sabroe *et al.*, 2003). Additionally, macrophages were shown to be a potent source of PGE2, reaching a constant level that is maintained at approximately 90 ng/10^6 cells (Sergeeva *et al.*, 1997). To investigate whether the observed donor-independent fluctuations in constitutive PKA dependence might be accounted for by variability in the culture conditions, such as the varying presence of residual PBMC in the preparation, or autocrine PMN secretions, it was investigated, whether these factors could protect PMN from apoptosis through the action of PKA.

Previously, it was shown that culturing PMN at higher densities or total cell numbers significantly lowered apoptosis rates (Hannah *et al.*, 1998), independent of cell adhesion (i.e. β2-Integrin receptors) and cell-matrix interactions. This sort of variation might be introduced due to small changes in the PMN concentration.

4.1.2.1. Influence of Paracrine and Autocrine Signalling on PMN Survival.

Therefore, highly pure PMN were incubated with or without 2 % PBMC for 4 hours and the effect on PKA-dependent PMN survival was examined by the addition of 0.7 mM Rp-8-Br-cAMPS. The presence of PBMC alone led to a low, non-significant increase in PMN survival (**Fig. 31A**). However, the high increase in PMN apoptosis induced by 0.7 mM Rp-8-Br-cAMPS in ultrapure PMN was significantly reduced in presence of 2% PBMC.

Thereafter, the influence of a putative autocrine signal was examined, which might be altered by fluctuations in cell culture density. In a supernatant transfer assay, PMN were stimulated with or without dbcAMP for 1 hour, and supernatants were collected, as described in chapter 2. PMN from the same donor were thereafter treated with media or PMN supernatant and

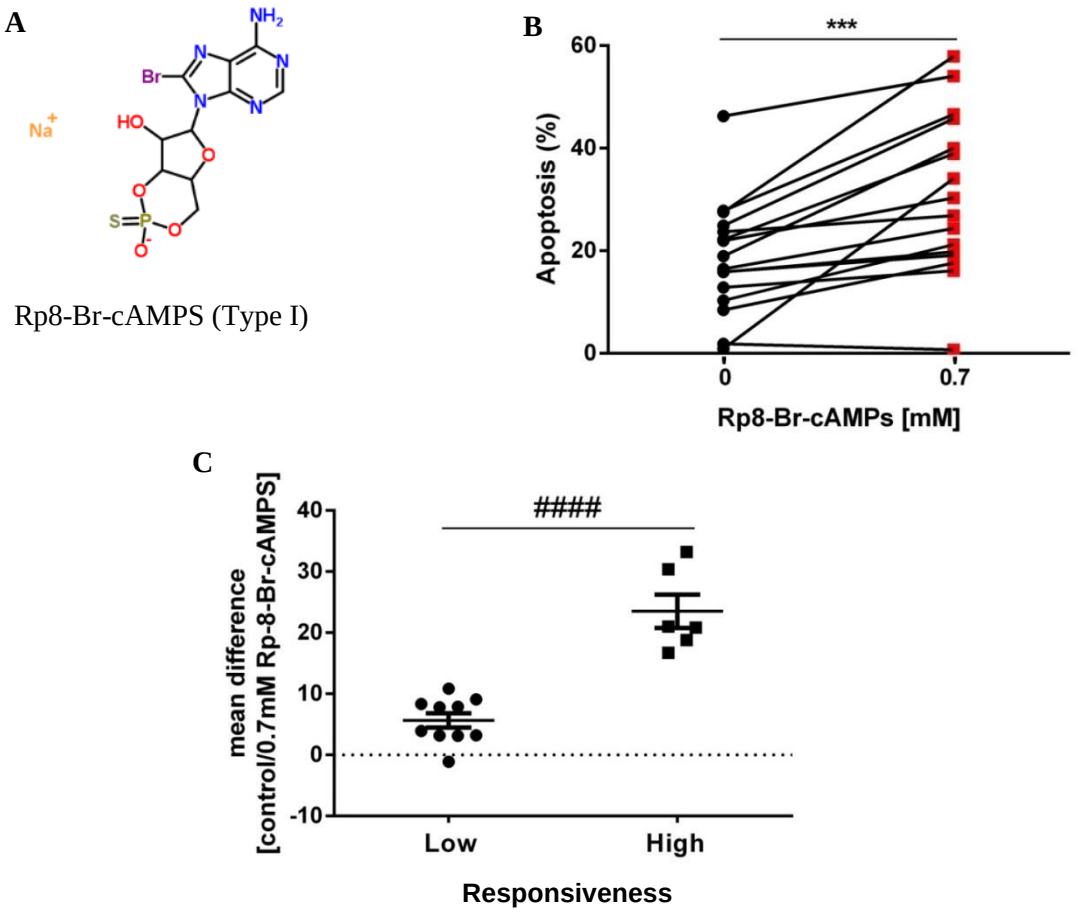


Figure 30: ***Competitive Inhibition of PKA Type I by Rp-8-Br-cAMPs Accelerates Constitutive PMN Survival.*** PMN were separated from whole blood through Percoll density gradient centrifugation and negative magnetic selection. Following isolation, PMN were cultured at 1.67 × 10^6/ml in RPMI and 10% FCS. Incubation in presence of 0.7 mM of the PKA Type I site selective antagonist Rp-8-Br-cAMPS (red squares) significantly increased PMN apoptosis at 4 hours post-purification (Panel **A**). In a proportion of experiments, a significantly higher responsiveness to PKA-inhibition by Rp-8-Br-cAMPS was observed in comparison to low responsive assays (Panel **C**). Data shown are individual datapoints and means ± SEM pooled from 16 independent experiments, as described in chapter 2.5. morphology was assessed by light microscopy. Statistical analysis was performed through a paired t-test. Asterisks (*) indicate differences to the control condition, whereas octothorpes (#) denote differences between low and high responsiveness to Rp-8-Br-cAMPS treatment. Results were considered to be statistically significant for $p<0.001$ (***) and $p<0.0001$ (####).

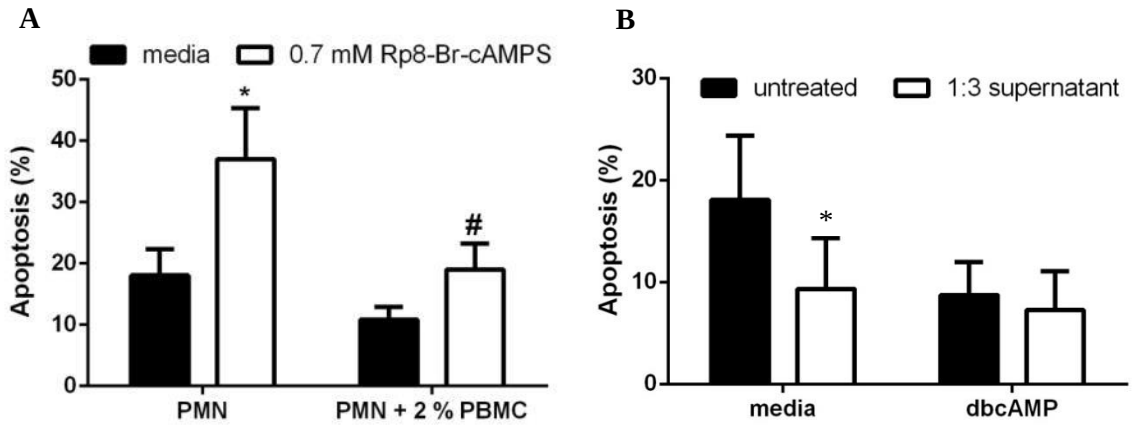


Figure 31: ***Paracrine and Autocrine Regulation of Basal PKA Survival in PMN.*** Ultrapure PMN with (Panel **A**) or without (Panel **B**) addition of 2 % PBMC were cultured for 4 hours in presence (Panel **A**) or absence (Panel **A**, **B**) of the competitive PKA Type I inhibitor Rp-8-Br-cAMPS (0.7 mM). For the PMN supernatant assay (Panel **B**), complete media was incubated for 1 hour in presence or absence of PMN, until supernatants were collected. PMN from the same donor were cultured with supernatants (1:3) for 3 hours (Panel **B**) in presence or absence of dbcAMP (50 μM). Basal PKA dependent PMN survival is largely independent of PBMC presence (Panel **A**). PMN supernatant treatment prolonged basal PKA survival through an autocrine stimulus (Panel **B**). Data shown are mean ± SEM of 4 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttest. Asterisks (*) denote significant differences to untreated conditions and octothorpes (#) indicate differences in Rp-8-Br-cAMPS-induced apoptosis in presence and absence of PBMC. Results were considered to be statistically significant for $p<0.05$ (*/#).

maintained in culture for a further 3 hours. Supernatant treatment significantly reduced PMN apoptosis by close to 50 % (**Fig. 31B**). Interestingly, supernatant reduced apoptosis to a similar extent as media, or supernatant with 50 μM dbcAMP, whereas there was no significant difference to supernatant treated PMN in presence of dbcAMP.

4.1.2.2. Regulation of Constitutive PMN Survival by Adenosine.

Adenosine is a well-known modulator of constitutive PMN survival (Yasui, *et al.*, 2000), and metabolic products of adenosine previously delayed PMN apoptosis via A2A/PKA (Pliyev *et al.*, 2014). To pursue the possibility that autocrine adenosine might enhance constitutive survival, the influence of adenosine and A2A receptors on PMN survival was examined. Highly pure PMN were incubated with increasing amounts of adenosine (0.1 -10 μM) for 4 hours. Adenosine significantly prolonged constitutive survival in a dose response curve at 1 μM and 10 μM (**Fig. 32A**).

Moreover, highly pure PMN were pretreated with or without the A2A receptor antagonist ZM 241385 (1 μM), or the PKA antagonist Rp-8-Br-cAMPS (0.7 mM). PMN survival induced by 10 μM adenosine was partially inhibited by A2A receptor antagonism by ZM 241385 (**Fig. 33A**) and completely abolished by PKA inhibition with Rp-8-Br-cAMPS (**Fig. 32B**).

When constitutive PMN survival was targeted by incubation with ZM 241385 alone, ZM 241385 dose-dependently accelerated PMN apoptosis (**Fig. 33B**). Addition of Rp-8-Br-cAMPS significantly increased constitutive survival, but did not have additional effects on rates of apoptosis when compared with ZM 241385 alone (**Fig. 33C**).

4.2. The Mechanisms of PGE2 Signalling in the Context of Inflammation.

Having established the importance of the prostaglandin receptor EP2 in PGE2 mediated PMN survival in chapter 3, it was queried, whether PGE2 induced survival via EP2 is PKA dependent. It was hypothesised that stimulation of EP2 by PGE2 affects PMN survival through cAMP/PKA signalling, as the EP2 receptor is coupled to stimulation of AC. Pretreatment with LPS previously reduced PGE2 induced cAMP elevation by 50 % (Okonogi *et al.*, 1991). Therefore, the effect of LPS presence on PKA mediated PMN survival and the influence of PBMC contamination in this setting was determined.

In a neutrophil microarray performed by the Sabroe/Whyte group, PKA activation by the selective PKA agonist pair N6/8-AHA, but not stimulation with LPS, caused a marked upregulation of the mRNA coding for the transcription factors NR4A2 and NR4A3 (unpublished data). NR4A receptors contribute to the regulation of survival in many cell types, and in fact, PGE2 was previously shown to induce NR4A gene expression through CREB (Holla *et al.*, 2006). However, the role of NR4A nuclear receptors in the regulation of PMN

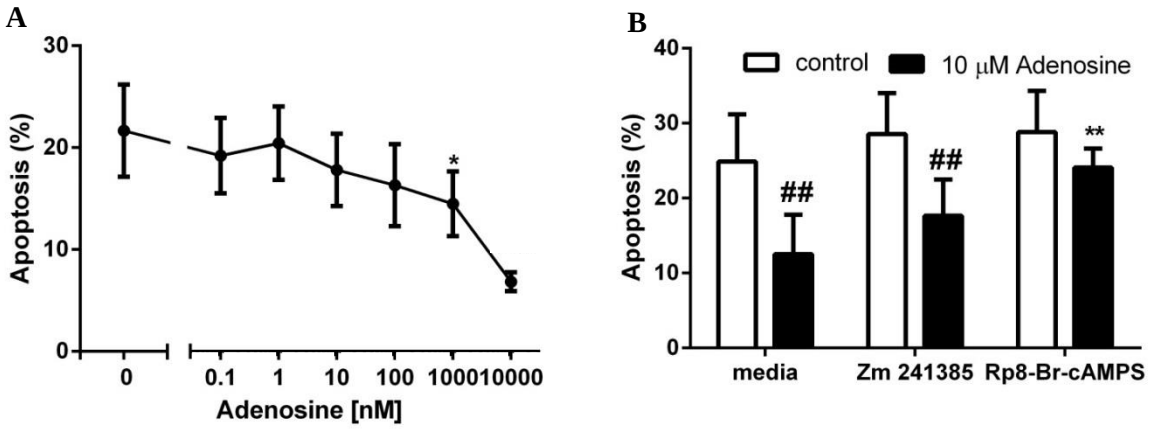


Figure 32: ***Adenosine Induced PMN Survival is Reversed by Rp8-Br-cAMPS and Partially Inhibited by A2A Receptor Inhibition.*** PMN were separated from whole blood through Percoll density gradient centrifugation and negative magnetic selection. Following isolation, PMN were cultured at 1.67×10^6/ml in RPMI and 10% FCS. Ultrapure PMN were pretreated for 15 minutes with either 1 μM of the A2A antagonist Zm 241385 or 0.7 mM of the PKA antagonist Rp8-Br-cAMPS (Panel **B**) and subsequently incubated for 4 hours in presence or absence of 10 μM adenosine (Panels **A**, **B**). White bars are control conditions, while black bars indicate incubation in the presence of 10 μM of adenosine (Panel **B**). Adenosine significantly delayed PMN apoptosis (Panels **A**, **B**), which was reversed by treatment with the PKA inhibitor Rp-8-Br-cAMPS and attenuated by the A2A antagonist Zm241385 (Panel **B**). Data shown are mean ± SEM of 3 (Panel **B**), or 4 (Panel **A**) independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Statistical analysis was performed through One Way RM ANOVA with Dunnet's posttest (Panel **A**) or Two Way RM ANOVA with Sidak's posttest (Panel **B**). Asterisks (*) indicate differences to control conditions, while octothorpes (#) denote differences in apoptosis between control and adenosine treated conditions. Results were considered to be statistically significant for $p<0.05$ (*) and $p<0.01$ (**/##).

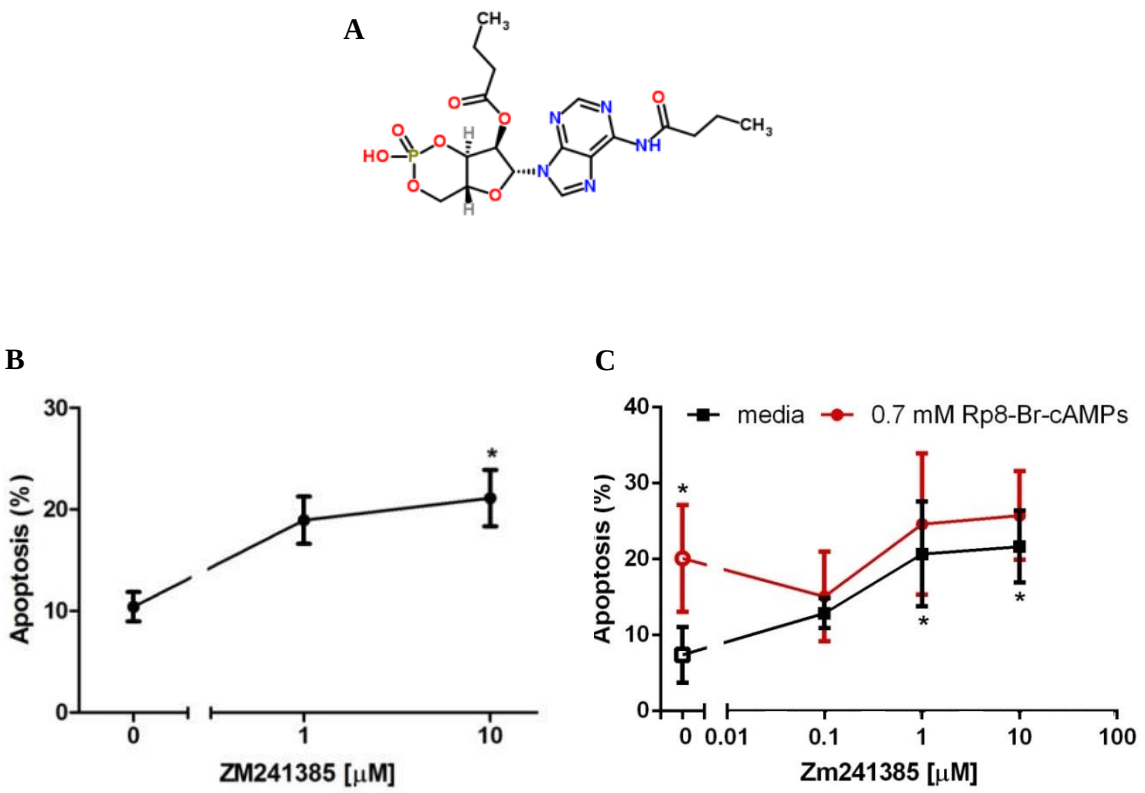


Figure 33: ***Basal Autocrine Signalling is Governed by Adenosine Signalling in PMN.*** Ultrapure PMN were cultured for 4 hours in presence (Panel **C**) or absence (Panel **B**) of the competitive PKA Type I inhibitor Rp-8-Br-cAMPS (0.7 mM) and with or without 0.1 - 10 μM of the A2A receptor antagonist ZM241385 (Panels **A-C**). Treatment with ZM241385 accelerated constitutive PMN apoptosis concentration dependently (Panel **B**, **C**). ZM 241385 abrogated basal PKA dependence to a similar magnitude as Rp-8-Br-cAMPS (Panel **C**). Data shown are mean ± SEM of 3 independent experiments (Panel **B**) or 4 independent experiments (Panel **C**) with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Statistical analysis was performed through One Way RM ANOVA with Dunnet's posttest (Panel **B**) or Two Way RM ANOVA with Sidak's posttest (Panel **C**). ***** denote significant differences to untreated conditions. Results were considered to be statistically significant for $p<0.05$ (*).

survival has not yet been determined, and thus the working hypothesis was here that PGE2/EP2 signalling induces PMN survival through the involvement of NR4A2 and NR4A3.

4.2.1. Regulation of PGE2 Survival through an EP2/PKA Signalling Axis.

To further substantiate the role of PKA in PGE2 survival, the influence of the PDE4 inhibitor rolipram in combination with PGE2 was investigated. For this end, highly purified PMN were incubated in presence or absence of 1 - 10 μM PGE2, with or without addition of 50 - 100 nM rolipram for 4 hours. Constitutive PMN survival was not significantly enhanced by PDE4 treatment in this assay (**Fig. 34A**). In the presence of PGE2, rolipram potentially decreased PMN apoptosis by a small, but non-significant extent, comparable to its influence on constitutive PMN apoptosis.

In an additional assay, highly purified PMN were incubated in the presence or absence of 1 μM PGE2, with or without addition of 100 nM rolipram for 4 – 20 hours to identify late effects of PDE4 activity. As before (**Fig. 34A**), addition of rolipram did not have a significant effect on constitutive PMN survival. However, while survival induced by 1 μM PGE2 was not significant in this assay, addition of 100 nM rolipram shifted the non-significant survival for PGE2 to a significant level (**Fig. 34B**). At 20 hours, the effect of PGE2 and rolipram on PMN apoptosis was lost.

Constitutive PKA signalling was active to a low degree in most PMN preparations, whereas PKA inhibition had a higher effect on PMN apoptosis in some assays (**Fig. 30**). PKA is known to play a role in PGE2 signalling and it was investigated whether PGE2-mediated survival is reversible by the PKA inhibitor Rp-8-Br-cAMPS. Highly pure PMN were simultaneously treated with increasing concentrations of PGE2 and the PKA antagonist Rp-8-Br-cAMPS to determine the level of PKA dependent PGE2 survival. In this set of experiments, 10 nM – 10 μM PGE2 induced PMN survival at 4 hours (**Fig. 35A, C**). Rp-8-Br-cAMPS [0.7 mM] increased constitutive apoptosis (**Fig. 35A**) and furthermore entirely blocked PMN survival induced by 10 nM PGE2, reaching statistical significance at concentrations of 100 nM to 10 μM (**Fig. 35A, C**).

Thereafter, it was aimed to verify the hypothesis of chapter 3; supporting a PKA-dependent EP2 phenotype in PGE2 induced PMN survival. Accordingly, highly pure PMN were treated with 100 nM and 1 μM of the EP2 agonist butaprost, with or without addition of 0.7 mM of the PKA antagonist Rp-8-Br-cAMPS. Butaprost alone induced PMN survival at 1 μM (**Fig. 35B, D**). Rp-8-Br-cAMPS not only increased constitutive apoptosis (**Fig. 35B**), but also significantly blocked butaprost-induced survival (**Fig. 35D**).

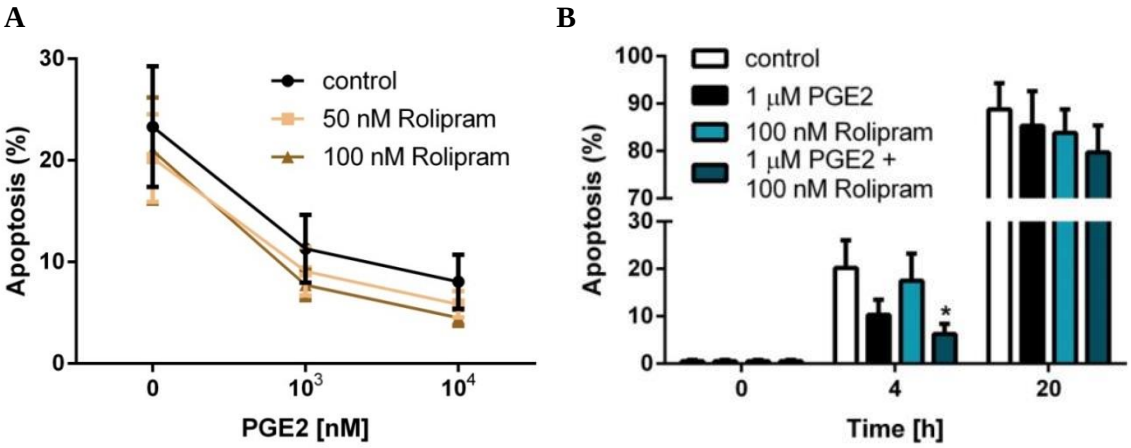


Figure 34: ***PMN Survival by High Concentrations of PGE2 is Enhanced by Phosphodiesterase Inhibition.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). Ultrapure PMN were preincubated for 15 minutes in presence or absence of 50 (Panel **A**), or 100 nM (Panel **B**) of the PDE4 inhibitor rolipram before incubation of 1 – 10 μM PGE2 for 4 (Panel **A**) or 20 hours (Panel **B**). PGE2 treatment non-significantly decreased PMN apoptosis at 4 hours (Panels **A**, **B**). In presence of 1 μM PGE2, rolipram significantly extended PMN survival at 4 hours (Panel **B**). Statistical analysis was performed through Two Way ANOVA with Sidak's posttests. Data shown are mean ± SEM of 3 (Panel **B**) or 4 (Panel **A**) independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Asterisks (*) indicate differences to the control condition. Results were considered to be statistically significant for $p<0.05$ (*/#).

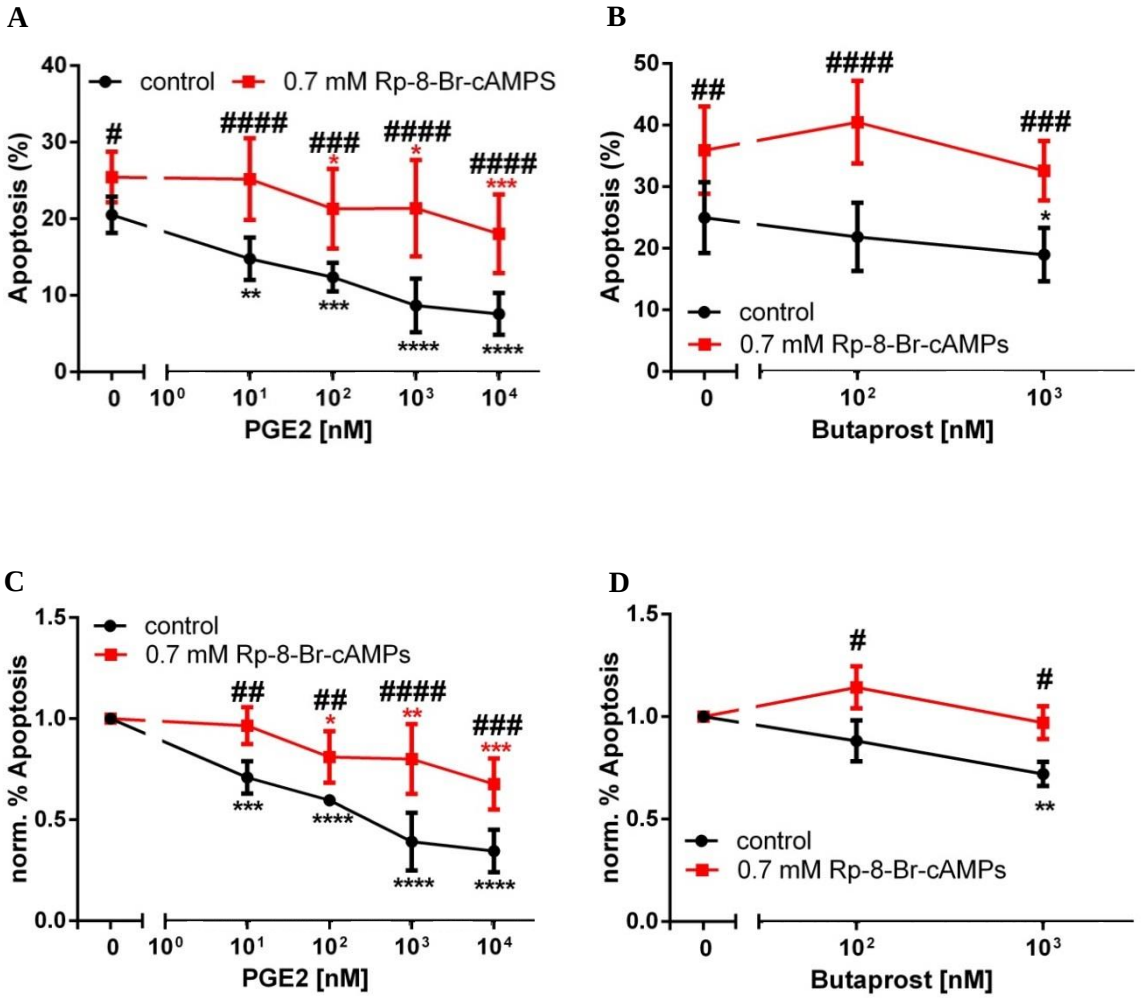


Figure 35: ***EP2 Receptor Agonist Butaprost Induces Rp8-Br-cAMPs Reversible PMN Survival.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). Ultrapure PMN were preincubated for 15 minutes in presence or absence of 0.7 mM of the PKA antagonist Rp-8-Br-cAMPS. Thereafter, 10 nM – 10 µM PGE2 (Panels **A**, **C**) or 0.1 - 1 µM Butaprost (Panels **B**, **D**) were added for a further 4 hours. Rp-8-Br-cAMPS significantly increased basal survival (Panels A, B). Moreover, Rp-8-Br-cAMPS significantly increased survival elicited by PGE2 (Panel **C**), or Butaprost (Panel **D**). Statistical analysis was performed through Two Way ANOVA with Sidak's posttests. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Asterisks (*) indicate differences to control conditions. Octothorpes (#) denote differences between treatments with and without Rp8-Br-cAMPS. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##), $p<0.001$ (***/###) and $p<0.0001$ (****/####).

4.2.2. Influence of PBMC on PKA dependent PGE2 Survival.

To investigate whether the presence of contaminating monocytes might account for the observed fluctuations in constitutive PKA dependence, highly purified PMN were treated with PGE2 and with or without Rp-8-Br-cAMPS for 4 and 20 hours. As previously shown (**Fig. 31A**), PMN survival was increased in the presence of PBMC (**Fig. 36A, B**). In both Percoll pure and highly pure PMN, PGE2 induced PKA dependent survival at 4 hours. As previously shown (**Fig. 12**), PGE2 survival was lost at 20 hours in ultrapure PMN (**Fig. 36A, B**). Similarly, PKA inhibition by Rp-8-Br-cAMPS did not significantly increase PMN apoptosis in highly pure PMN (**Fig. 36B**). Interestingly, in Percoll pure PMN, PGE2 induced a non-significant reduction in late PMN apoptosis that was significantly blocked by Rp-8-Br-cAMPS, which considerably increased PMN apoptosis at 20 hours (**Fig. 36A**).

4.2.3. Interdependence of LPS and PGE2 Signalling in PKA Survival Pathways in PMN.

In chapter 3.2, the effect of LPS-induced EP2 and EP4 receptor expression on PMN survival was examined. Moreover, it was shown in chapter 4.2.1 that EP2 mediated PMN survival is dependent on PKA activation. To further examine the molecular mechanisms involved in the potential synergy of LPS and PGE2 signalling, the PKA dependence of LPS and its potential effects on PGE2 survival was examined in Percoll pure and highly pure PMN. For this, highly pure or Percoll pure PMN were incubated with LPS, PGE2 or both for 4 hours. In both cell types, Rp-8-Br-cAMPS increased constitutive PMN apoptosis. LPS inhibited PMN apoptosis in highly pure PMN (**Fig. 37B**) and in Percoll pure PMN (**Fig. 37A**). In ultrapure (**Fig. 37B**), but not Percoll pure PMN (**Fig. 37A**), PGE2 significantly delayed apoptosis, which was reversed by Rp-8-Br-cAMPS treatment. Treatment with both LPS and PGE2 significantly reduced PMN apoptosis in both assays (**Fig. 37A, B**). Interestingly, in both Percoll pure and ultrapure PMN, Rp-8-Br-cAMP-induced apoptosis in presence of both PGE2 and LPS was comparable to the effect of the antagonist in presence of LPS alone. To further validate a role for PKA in LPS and PGE2-mediated PMN survival, highly pure PMN were incubated a range of LPS concentrations (10 pg/ml - 10 ng/ml) in the presence or absence of Rp-8-Br-cAMPS. LPS profoundly delayed PMN apoptosis, reaching statistical significance at 100 pg/ml (**Fig. 38A, C**). Interestingly, Rp-8-Br-cAMPS increased constitutive PMN apoptosis, but the effect of the antagonist was diminished with increasing LPS concentrations (**Fig. 38A**). Moreover, the normalisation of data in **Fig. 38A** revealed a PKA-independent effect of LPS on PMN survival (**Fig. 38C**).

Thereafter, PMN were coincubated with increasing amounts of PGE2 (10 nM – 10 μM) and 100 pg/ml LPS, in the presence or absence of Rp-8-Br-cAMPS. Simultaneous treatment with PGE2 (10 nM - 10 μM) and LPS [100 pg/ml] inhibited PMN apoptosis, reaching statistical significance at 1 μM (**Fig. 38B, D**). However, in contrast to PGE2 alone (**Fig. 35A, C**),

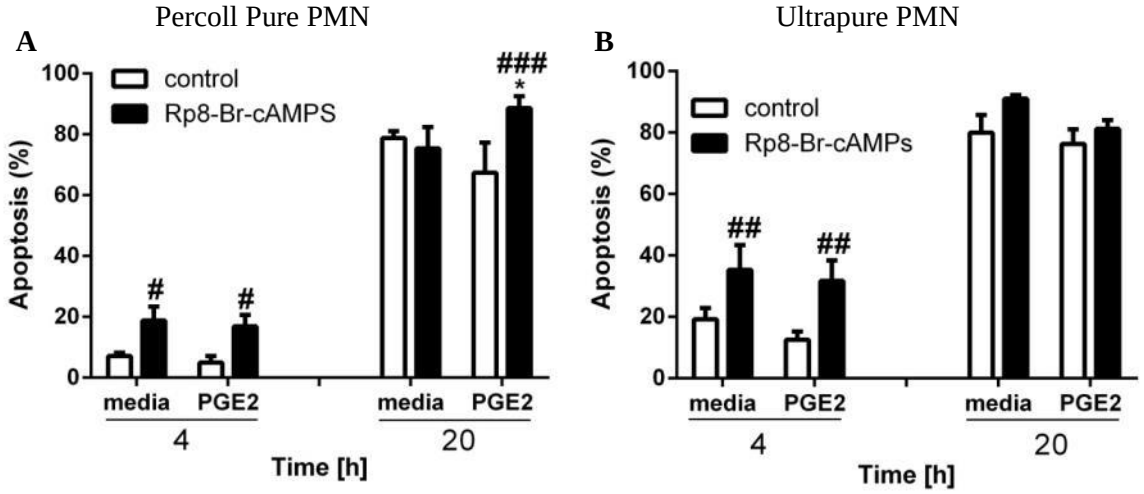


Figure 36: ***Temporal Regulation of PMN Apoptosis by PGE2-Initiated Engagement of PKA Signalling.*** Percoll purified PMN (Panel **A**) were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %; Panel **B**). PMN were preincubated for 15 minutes in presence or absence of 0.7 mM of the PKA antagonist Rp-8-Br-cAMPS. Thereafter, PMN were incubated for 4 or 20 hours in presence of 10 μM PGE2. Rp-8-Br-cAMPS significantly increased constitutive apoptosis in both Percoll pure (Panel **A**) and highly pure PMN (Panel **B**). In this assay, PGE2 did not significantly induce PMN survival. However, in Percoll pure PMN only, the effect of Rp-8-Br-cAMPS on apoptosis was maintained in presence of PGE2 at 20 hours (Panel **A**). Statistical analysis was performed through Two Way ANOVA with Sidak's posttests. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Asterisks (*) denote differences to control. Octothorpes (#) indicate differences between treatments with and without Rp8-Br-cAMPS. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (##) and $p<0.001$ (###).

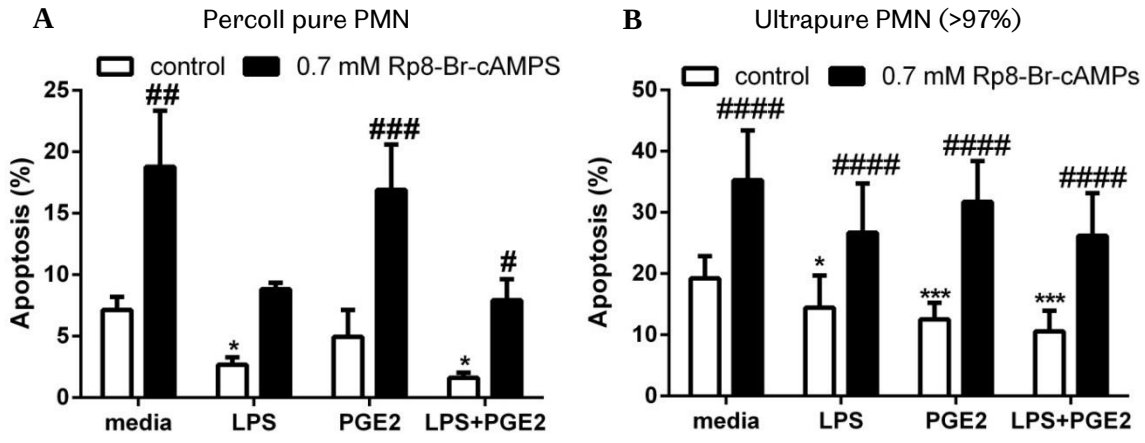


Figure 37: ***Regulation of PMN Survival by LPS and PGE2: Influence of Cell Purity.*** Percoll purified PMN (Panel **A**) were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %; Panel **B**). PMN were preincubated for 15 minutes in presence or absence of 0.7 mM of the PKA antagonist Rp-8-Br-cAMPS. Thereafter, PMN were incubated for 4 or 20 hours in presence of 1 ng/ml LPS, 10 μM PGE2, or both. Rp-8-Br-cAMPS significantly increased constitutive apoptosis in both Percoll pure (Panel **A**) and highly pure PMN (Panel **B**). LPS increased PMN survival in a partially PKA-dependent manner in both assays. Here, PGE2 increased PMN survival in ultrapure (Panel **B**), but not Percoll pure PMN (Panel **A**). Interestingly, the effect of PGE2 on PMN survival was reduced in percoll pure PMN (Panel **A**). Statistical analysis was performed through Two Way ANOVA with Sidak's posttests. Data shown are mean ± SEM of 4 (Panel **A**) or 5 (Panel **B**) independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Asterisks (*) denote differences to control. Octothorpes (#) indicate differences between treatments with and without Rp-8-Br-cAMPS. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (##), $p<0.001$ (***/###) and $p<0.0001$ (####).

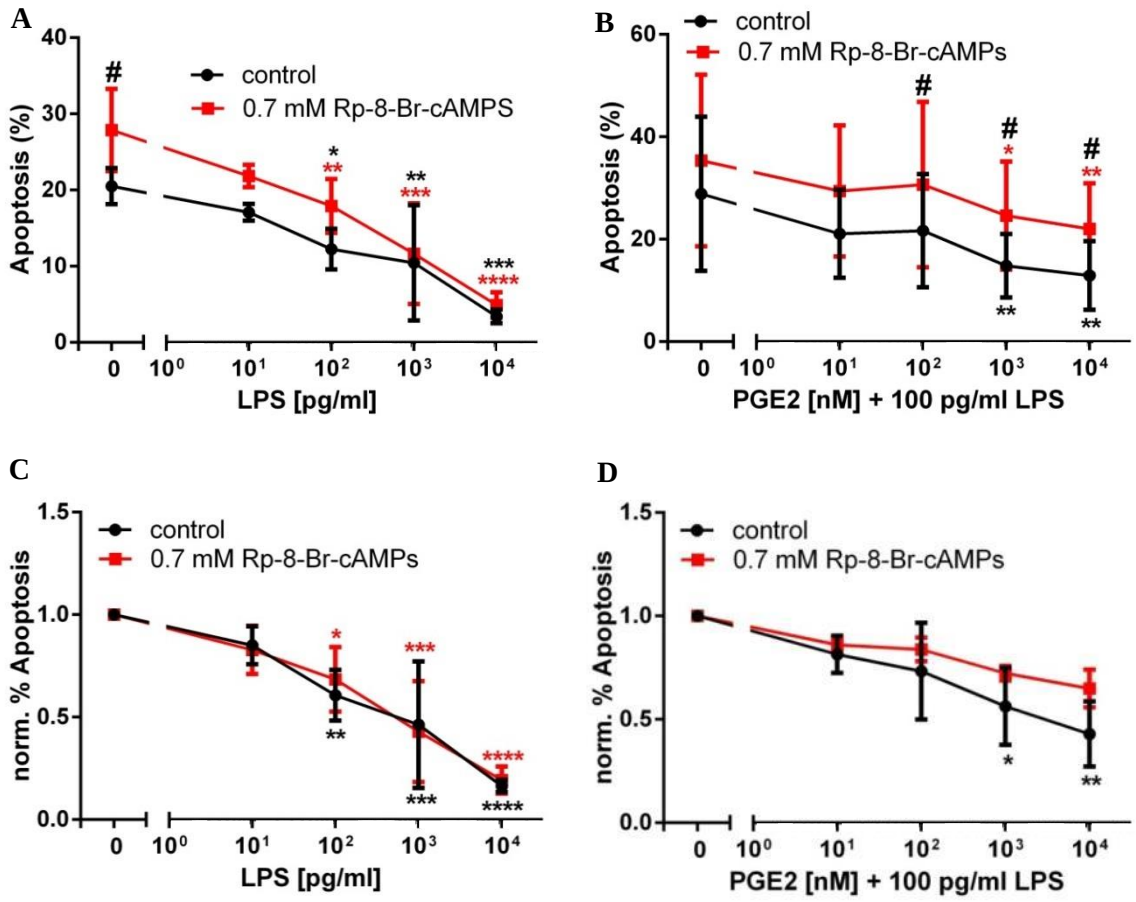


Figure 38: ***LPS Induces PKA-Independent PMN Survival in Absence of PGE2.*** Percoll purified PMN were further refined by negative magnetic selection with a custom antibody cocktail (purity > 97 %). PMN were pre-incubated for 15 minutes in presence (red lines) or absence (black lines) of the competitive PKA Type I inhibitor Rp8-Br-cAMPS. Thereafter, PMN were incubated with varying concentrations of PGE2 (0.01 - 10 µM) or LPS (0.01 - 10 ng/ml), as indicated. LPS (Panels **A, C**) treatment dose-dependently decreased apoptosis rates at 4 hours post inoculation. Rp-8-Br-cAMPS significantly delayed constitutive PMN survival, while Rp-8-Br-cAMPS induced apoptosis was dose-dependently abrogated in presence of LPS (Panels **A, C**). Incubation with both LPS and PGE2 increased PMN survival, which was increased to a constant level by Rp-8-Br-cAMPS treatment (Panels **B, D**). Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttest. Asterisks (*) denote significant differences to untreated conditions and octothorpes (#) indicate differences between Rp-8-Br-cAMPs treatment conditions. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****).

Rp-8-Br-cAMPS did not block the effect of PGE2 at higher concentrations, but mirrored the treatment curve for PGE2 and LPS, with only non-significant increases in Rp-8-Br-cAMPS-induced apoptosis with increasing PGE2 concentrations (**Fig. 38D**).

4.2.4. NR4A: Mechanism of PKA dependent PMN Regulation through Nuclear Receptors.

Therapeutic targeting of neutrophil survival is considered to be an attractive strategy in the regulation of neutrophillic inflammation *in vivo*. NR4A2 and NR4A3 are orphan transcription factors in the nuclear receptor family, and are implicated in cell survival pathways in various cell types including macrophages. NR4A2 and NR4A3 are well-established components of cAMP/PKA signalling in neurons (Hawk, Abel, 2011) and Th1 cells (Yao *et al.*, 2012). Moreover, in gastrointestinal disorders, *NR4A2* expression is induced by PGE2, in a PKA-dependent manner (Holla *et al.*, 2006; Han *et al.*, 2012). However, their role in neutrophil survival in response to PGE2 and PKA activation is still largely unknown due to the short lifespan and genetic intractability of these immune cells. It was hypothesised that orphan nuclear receptor NR4A family members play a role in neutrophil cell survival.

In a human neutrophil microarray study previously conducted by Sabroe group members, expression of *NR4A2* and *NR4A3* was rapidly induced by the PKA agonist N6/8-AHA-cAMP (unpublished work). To validate this, highly pure PMN were incubated with or without dbcAMP (50 – 100 μM) for the indicated amount of time (**Fig. 39**). *NR4A2* and *NR4A3* mRNA expression dramatically decreased at an equal rate over the course of 20 hours of culture (**Fig. 39A, B**). Treatment with dbcAMP dose-dependently increased *NR4A2*/*NR4A3* expression levels at 4 hours (**Fig. 39C**) and 20 hours (**Fig. 39D**). In the same assay, 100 μM dbcAMP restored *NR4A3* expression to levels comparable to constitutive expression (**Fig. 39B**).

Highly pure PMN were incubated with the cAMP-elevating agent adenosine, the A2A antagonist Zm 241385 and Rp-8-Br-cAMPS. Similar to **Fig. 39**, *NR4A2* and *NR4A3* mRNA expression increased by qPCR upon adenosine treatment, and was inhibited by 0.7 mM Rp-8-Br-cAMPS at 4 hours (**Fig. 40A, B**). Zm 241385 attenuated adenosine-induced *NR4A2* (**Fig. 40A**), but not *NR4A3* (**Fig. 40B**) expression in the same assay.

Thereafter, highly pure PMN were pretreated for 15 minutes with 0.7 mM Rp-8-Br-cAMPS, and subsequently incubated with or without PGE2 for the indicated amount of time. *NR4A2* and *NR4A3* mRNA expression was examined. Treatment of highly pure PMN with PGE2 resulted in upregulation of *NR4A2* (**Fig. 41A**) and *NR4A3* (**Fig. 41B**) at 8 hours, the latter of which was inhibited by Rp-8-Br-cAMPS. LPS did not significantly alter *NR4A2* and *NR4A3* mRNA expression (**Fig. 42A, B**). Interestingly, treatment with both LPS and PGE2 further potentiated *NR4A2* and *NR4A3* expression (**Fig. 42A, B**), and was partially inhibited by Rp-8-Br-cAMPS

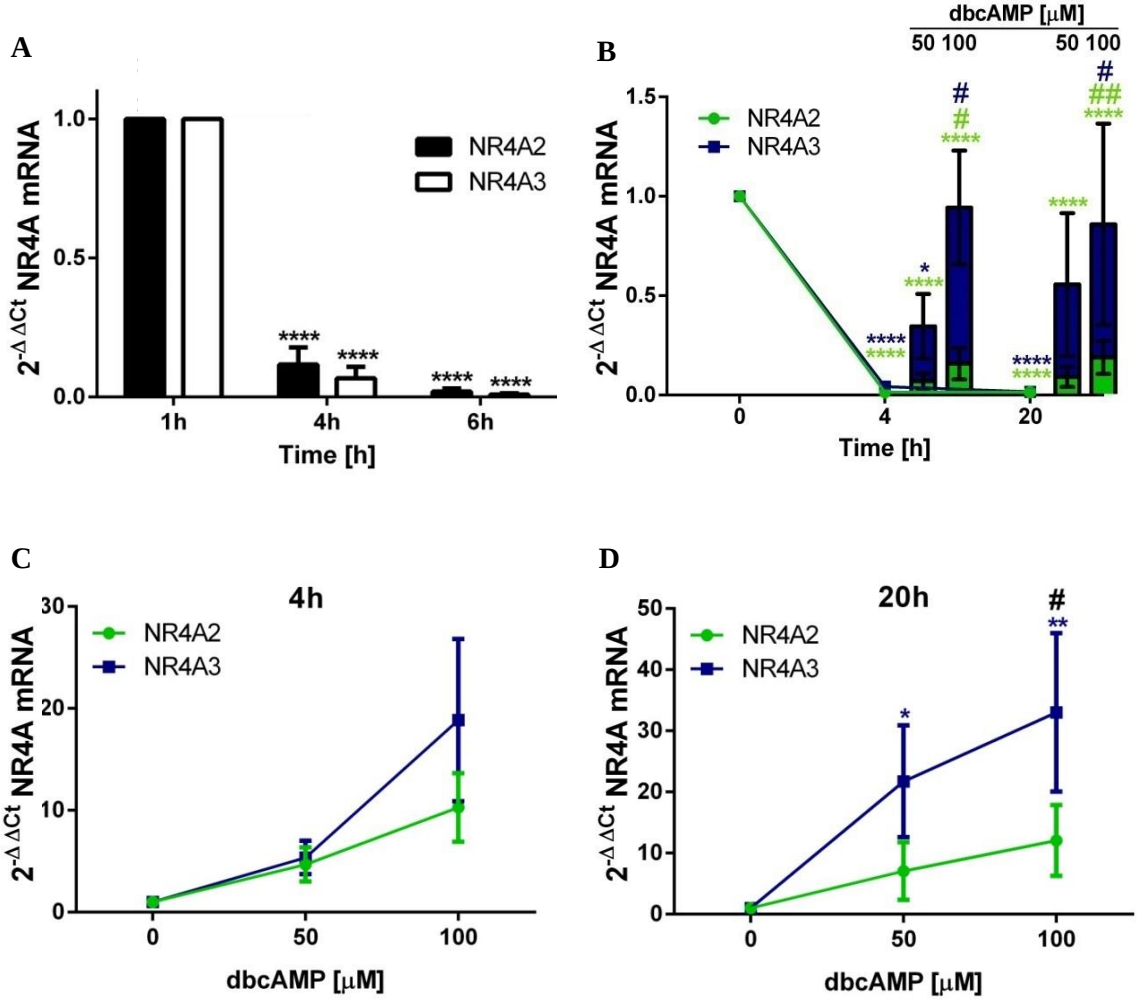


Figure 39: ***dbcAMP Induces NR4A2 and NR4A3 mRNA Expression in PMN.*** Ultrapure PMN were incubated for 0 – 20 hours (as indicated) in presence or absence of 50 – 100 μM of the PKA agonist dbcAMP. mRNA was extracted using the TRI method (as described in chapter 2.3.1.). Total cellular RNA was reverse transcribed into cDNA using random primers. Resulting cDNA was then analysed by qPCR using primers for *NR4A2*, *NR4A3* and *GAPDH* as a normalisation control. *NR4A2* and *NR4A3* mRNA expression was significantly reduced at 4 hours compared to 1 hour (Panel **A**) or 0 hour (Panel **B**) timepoints. In the same assay as Panel **B**, treatment with dbcAMP dose-dependently increased NR4A expression levels at 4 hours (Panel **C**) and 20 hours (Panel **D**). 100 μM dbcAMP restored *NR4A3* expression to levels comparable to constitutive expression (Panel **B**). Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. qPCR expression levels were normalised by using a *GAPDH* standard curve. Asterisks (*) denote differences to 1 hour (Panel **A**), 0 hour (Panel **B**) or untreated conditions (Panel **C** and **D**). Octothorpes (#) indicate differences between *NR4A2* and *NR4A3* mRNA expression. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (**/##) and $p<0.0001$ (****).

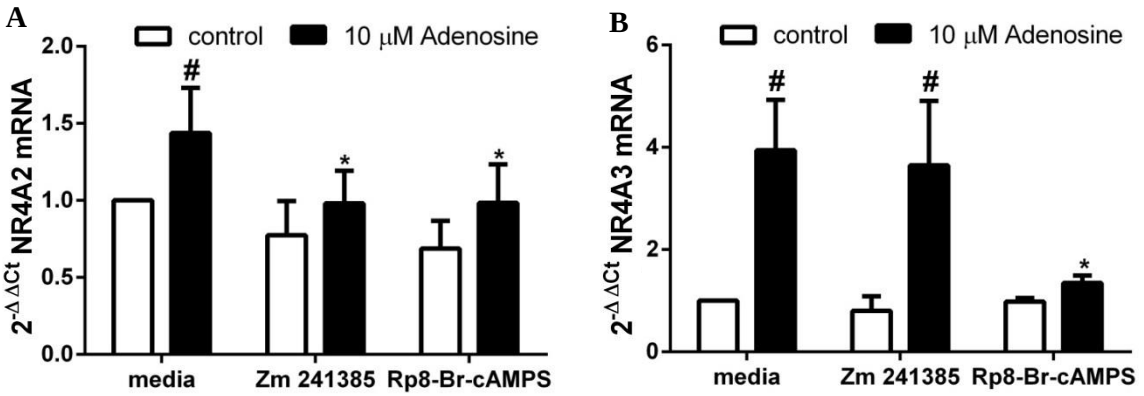


Figure 40: ***Adenosine Induces PKA-Dependent NR4A2 and NR4A3 mRNA Expression.*** Ultrapure PMN were pretreated for 15 minutes with either 1 μM of the A2A antagonist Zm 241385 or 0.7 mM of the PKA antagonist Rp8-Br-cAMPs and subsequently incubated for 4 hours in presence or absence of 10 μM adenosine. mRNA was extracted using the TRI method (as described in chapter 2.3.1.). Total cellular RNA was reverse transcribed into cDNA using random primers. Resulting cDNA was then analysed by qPCR using primers for *NR4A2* (Panel **A**), *NR4A3* (Panel **B**) and *GAPDH* as a normalisation control. Adenosine increased NR4A2 and NR4A3 expression, which was abolished by Rp8-Br-cAMPs treatment. A2A antagonist Zm 241385 attenuated Adenosine-induced NR4A2 (Panel **A**), but not NR4A3 (Panel **B**) expression. Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests. Data shown are mean ± SEM of 4 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. qPCR expression levels were normalised by using a *GAPDH* standard curve. Asterisks (*) denote differences to control conditions. Octothorpes (#) indicate differences of *NR4A* mRNA expression in presence and absence of adenosine. Results were considered to be statistically significant for $p<0.05$ (*/#).

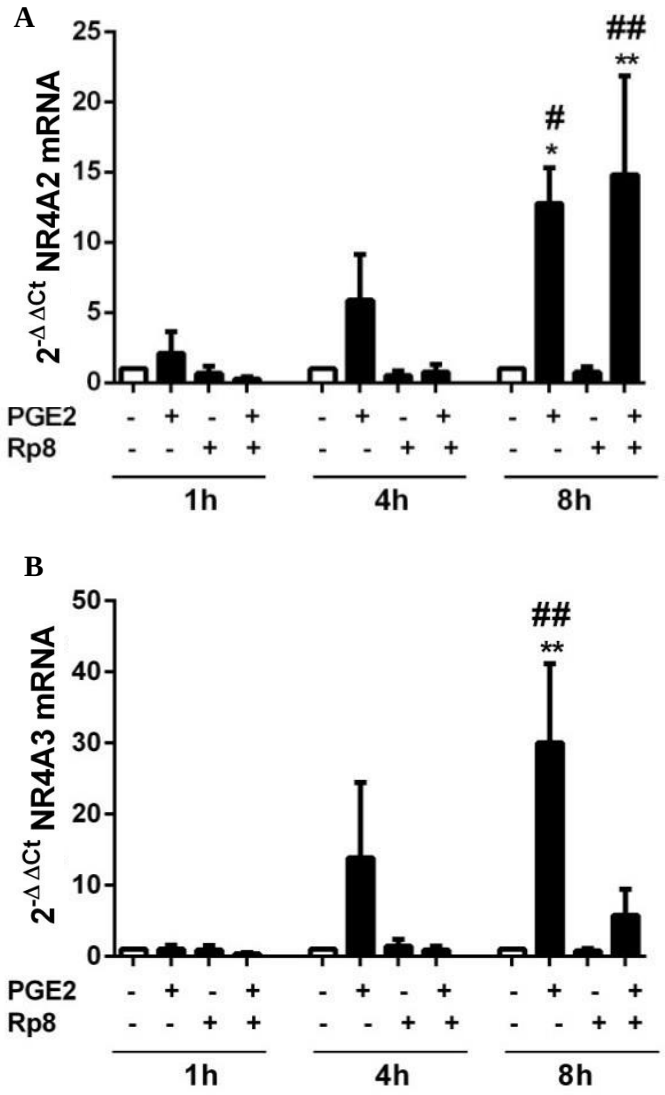


Figure 41: ***Temporal Regulation of NR4A2 and NR4A3 mRNA Expression by PGE2.*** Ultrapure PMN were incubated for 1 - 8 hours. PMN were pretreated for 15 minutes with or without 0.7 mM Rp-8-Br-cAMPS before addition of 10 μM PGE2 (Panels **A**, **B**). mRNA was extracted using the TRI method (as described in chapter 2.3.1.). Total cellular RNA was reverse transcribed into cDNA using random primers. Resulting cDNA was then analysed by qPCR using primers for NR4A2 (Panel **A**), NR4A3 (Panel **B**) and GAPDH as a normalisation control. PGE2 robustly increased NR4A2 and NR4A3 expression. Upregulation of NR4A3 at 8 hours was inhibited by Rp-8-Br-cAMPS (Panel **B**). Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests. Data shown are mean ± SEM of 3 independent experiments (Panels **B**) with cells isolated from distinct healthy volunteers, as described in chapter 2.5. qPCR expression levels were normalised by using a *GAPDH* standard curve. Asterisks (*) denote significant differences to 1 hour incubation. Octothorpes (#) indicate differences to the 8 hour media control. Results were considered to be statistically significant for $p<0.05$ (*/#) and $p<0.01$ (**/##).

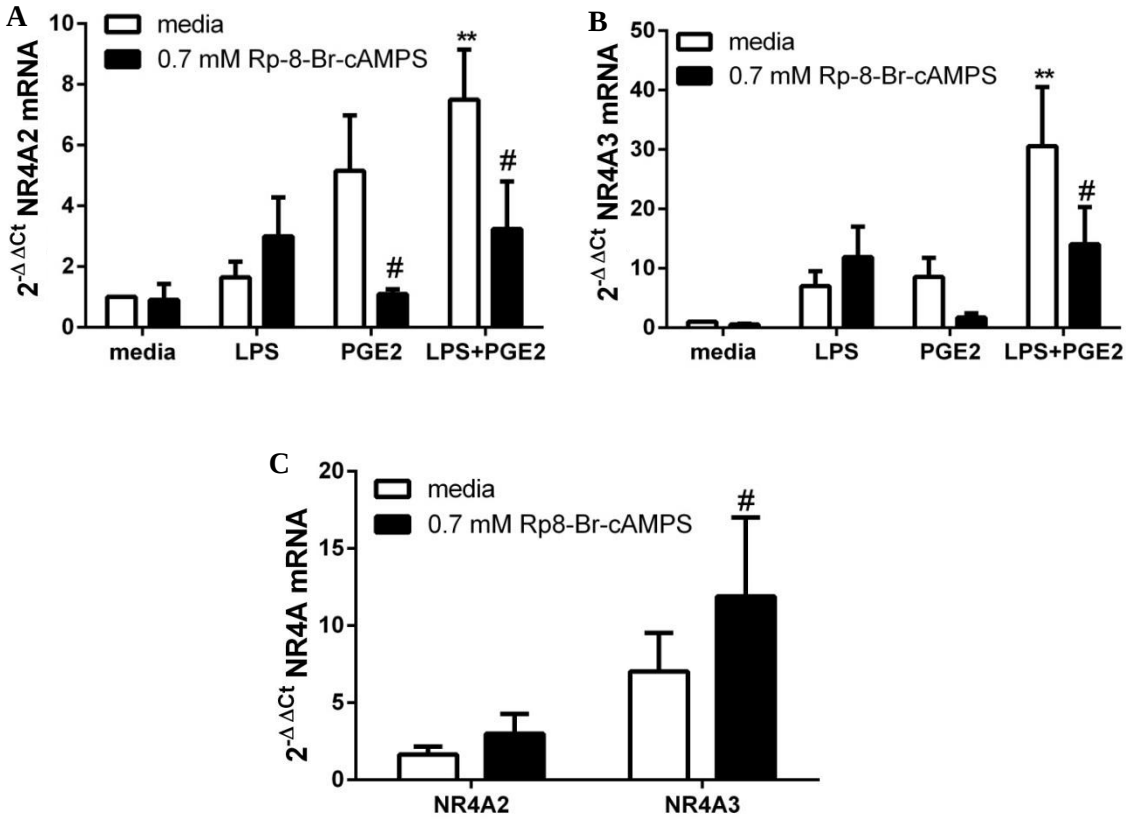


Figure 42: ***Synergistic Induction of NR4A3 Expression by LPS and PGE2 is Inhibited by Rp-8-Br-cAMPS.*** Ultrapure PMN were incubated for 4 hours. PMN were pretreated for 15 minutes with or without 0.7 mM of the PKA inhibitor Rp-8-Br-cAMPS before addition of 10 μM PGE2 (Panels **A**, **B**), 1 ng/ml LPS (Panels **A**-**C**) or a combination of both (Panels **A**, **B**). mRNA was extracted using the TRI method (as described in chapter 2.3.1.). Total cellular RNA was reverse transcribed into cDNA using random primers. Resulting cDNA was then analysed by qPCR using primers for NR4A2, NR4A3 and GAPDH as a normalisation control. PGE2 and LPS synergistically increased NR4A2 and NR4A3 expression (Panels **A, B**), which was inhibited by Rp-8-Br-cAMPS treatment. Rp-8-Br-cAMPS significantly increased LPS-induced NR4A3 expression (Panel **C**). The level of NR4A2 and NR4A3 expression was similar upon Rp-8-Br-cAMPS treatment in PMN treated with LPS alone, or both PGE2 and LPS (Panels **A**, **B**). Moreover, inhibition of PKA signalling stabilised *NR4A3* levels in presence of LPS (Panel **A**). Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests (Panels **A-C**). Data shown are mean ± SEM of 5 independent experiments with cells isolated from distinct healthy volunteers (Panels **A, B**), as described in chapter 2.5. Panel **C** shows a comparison of LPS treated cells only. qPCR expression levels were normalised by using a GAPDH standard curve. Asterisks (*) denote significant differences to the media control. Octothorpes (#) indicate differences between control and Rp-8-Br-cAMPS treated conditions. Results were considered to be statistically significant for $p<0.05$ (#) and $p<0.01$ (**/##).

treatment. Moreover, in a direct comparison of NR4A expression in LPS-treated cells only, Rp-8-Br-cAMPS significantly increased *NR4A3* expression in LPS-treated PMN with a similar tendency for *NR4A2* (**Fig. 42C**).

In chapter 3.1.2., a role for EP2 in mediating PGE2 survival was identified, therefore, the receptor involved in the upregulation of *NR4A2* and *NR4A3* by PGE2 was also examined. Highly pure PMN were incubated with 10 μM PGE2, the EP2 agonist butaprost, or the EP4 agonist L-902,688 for 4 hours, and the respective mRNA expression of *NR4A2* and *NR4A3* was evaluated by qPCR. PGE2 increased *NR4A2* expression 3.75 fold and *NR4A3* expression 17.86 fold (**Fig. 43A, B**). Similarly, butaprost partially induced *NR4A2* and *NR4A3* expression by a mean of 2.41 fold and 6.92 fold respectively. L-902,688 in contrast was inefficient at increasing *NR4A2* expression, and only induced a non-significant 1.74 mean fold increase in *NR4A3* expression.

To investigate the influence of LPS and PGE2 synergy in the context of chronic lung inflammation, Percoll pure PMN from healthy controls and COPD patients were incubated in the presence of LPS for 0 – 120 minutes, and *NR4A2* and *NR4A3* expression was measured by qPCR. LPS induced the expression of *NR4A3*, but not *NR4A2* in both healthy control and COPD patient PMN (**Fig. 44A**, **Fig. 45A**). Despite comparable mRNA expression at baseline of both *NR4A2* and *NR4A3* (data not shown), LPS mediated a comparatively high fold change of *NR4A3* mRNA from baseline in healthy control PMN (7.37 mean fold induction) and COPD PMN (8.32 mean fold induction), whereas *NR4A2* was only induced to a low fold extent by LPS at 2 hours in healthy control PMN (1.88 mean fold induction) and COPD PMN (2.51 mean fold induction). When incubating both types of PMN for 4 hours with or without LPS and PGE2, there were no detectable differences in *NR4A* expression between the cell types (**Fig. 44B**, **Fig. 45B**).

4.3. Discussion

4.3.1. PKA Extends PMN Survival.

PKA is a potent pro-survival mediator in PMN (Vaughan *et al.*, 2007). PKA activation can be achieved through the activation of AC or the inhibition of cAMP metabolism by PDE. In this chapter, the modulation of PKA activation was examined using the PDE4 inhibitor rolipram, the PKA agonist dbcAMP and the agonist pair N6-MB-cAMP/8-AHA-cAMP (N6/8-AHA), as well as the PKA antagonist Rp-8-Br-cAMPS.

In previous studies, the role of PGE2 in elevating intracellular cAMP levels and the connection to PKA-dependent PMN survival has already been established. Briefly, 10 μM PGE2 mediated a >50% increment in intracellular cAMP after 30 minutes incubation in PMN, as determined by a radioimmunoassay for intracellular cAMP accumulation (Ottonello *et al.*, 1998). In the same

study, 10 μM PGE2 inhibited PMN apoptosis by >30 % at 18 hours. A range of cAMP-elevating agents, including dbcAMP and forskolin, as well as various prostaglandin analogs, were found to significantly attenuate PMN apoptosis at 20 hours, assessed by CD16 expression (Rossi *et al.*, 1995). Moreover, PMN survival induced by 200 μM dbcAMP was blocked by treatment with 100 μM of the PKA inhibitor H-89 at 20 hours, confirming the involvement of cAMP in PKA activation in PMN apoptosis.

4.3.1.1. PDE4 Inhibition has a Limited Effect on PMN Survival.

Forskolin is the best established and most widely used AC agonist, which efficiently elevates intracellular cAMP (Truss *et al.*, 1996). However, its rather non-specific action, in particular towards glucose transporters, ion channels, further membrane transport proteins and the inhibition of Platelet activating factor (PAF) binding (Wong *et al.*, 1993), make it unsuitable for the use in the research of specific signalling pathways. Therefore, the selective PDE4 antagonist rolipram was employed in this study due to the reasons outlined below.

The efficiency of PDE inhibition likewise relies on intracellular cAMP stocks, as well as constitutive PDE activity. Agents that non-selectively inhibit PDE are IBMX and theophylline, both of which have been shown to inhibit adenosine receptors (Wu *et al.*, 1982; Daly *et al.*, 1987), and might therefore interfere with other PKA responsive pathways.

The predominant PDE subtype expressed in human leukocytes is PDE4 (Wang *et al.*, 1999) and rolipram is a widely used tool in PDE4 inhibition. Rolipram is highly selective towards human PDE4 subtypes A, B, C and D with Ki values of 1.1 ± 0.3 nM, 0.9 ± 0.0 nM, 324.6 ± 27.4 nM and 61.1 ± 12.8 nM respectively (Wang *et al.*, 1997). In human PMN, 50 nM rolipram inhibited the fMLP induced superoxide generation by 47 % (Talpain *et al.*, 1995), PMN chemotaxis with an EC50 of 23.0 ± 2.3 nM and it potentiated inhibition of chemotaxis by PGE2 with 57.2 ± 5.9 nM (Armstrong *et al.*, 1995). Moreover, rolipram showed considerably higher IC50 values ranging from 200 nM to 3 μM in various other cell types and species (amongst others: Tenor *et al.*, 1996; Souness *et al.*, 1994). However, rolipram was inefficient at elevating cAMP levels in SMC (Truss *et al.*, 1996). This might reflect inherent variations in the constitutive PDE4 activity in these cell types, which thus have to be empirically determined in every cell type.

Consequently, PMN were treated with a range of rolipram concentrations (1 nM – 10 μM), but rolipram was unable to significantly prolong PMN survival at any concentration tested. The lack of effect of rolipram may be due to low effect of constitutive PDE4 activity on PMN survival in the assays of this study (**Fig. 27**). At the two highest concentrations a limited, non-significant increase in PMN survival was observed, which was only 3 fold above the Ki of rolipram towards the PDE4 subtype C (Wang *et al.*, 1997), potentially suggesting a differential role for PDE4 subtypes in PMN survival. However, this finding might also be correlated with a

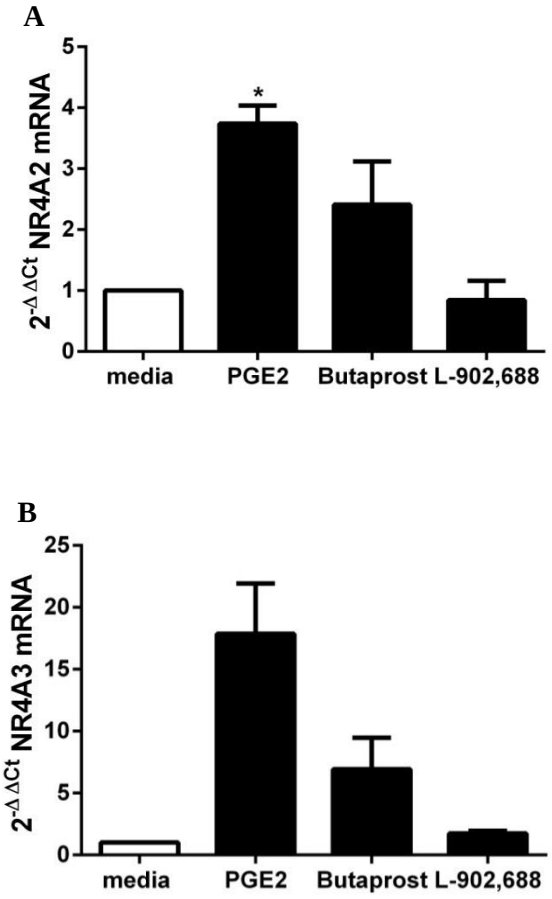


Figure 43: ***PGE2-Induced NR4A2 and NR4A3 Expression is Mimicked by EP2 Agonist Butaprost, but not EP4 Agonist L-902,688.*** Ultrapure PMN were incubated for 4 hours with 10 μM PGE2, Butaprost or L-902,688 for 4 hours. mRNA was extracted using the TRI method (as described in chapter 2.3.1.). Total cellular RNA was reverse transcribed into cDNA using random primers. Resulting cDNA was then analysed by qPCR using primers for *NR4A2* (Panel **A**), and *NR4A3* (Panel **B**) and *GAPDH* as a normalisation control. PGE2 substantially increased *NR4A2* and *NR4A3* expression. Butaprost similarly increased *NR4A2* and *NR4A3* expression, while L-902,688 was ineffective at increasing *NR4A* expression levels. Statistical analysis was performed through paired t-tests. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. qPCR expression levels were normalised by using a *GAPDH* standard curve. Asterisks (*) denote significant differences to the media control. Results were considered to be statistically significant for $p<0.05$ (*).

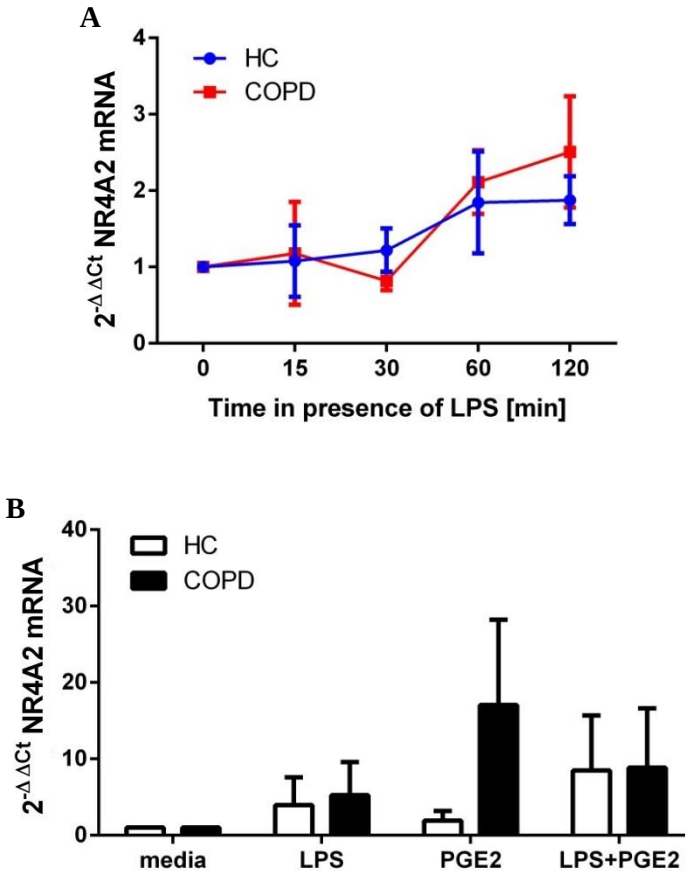


Figure 44: ***Effect of LPS and PGE2 on NR4A2 Expression in COPD PMN.*** Percoll pure PMN from healthy control and COPD patients were incubated for 0 – 120 minutes with 10 ng/ml LPS (Panel **A**), or 4 hours with 10 ng/ml LPS, 10 µM PGE2, or both for 4 hours (Panel **B**). mRNA was extracted using the TRI method (as described in chapter 2.3.1.). Total cellular RNA was reverse transcribed into cDNA using random primers. Resulting cDNA was then analysed by qPCR using primers for NR4A2 and GAPDH as a normalisation control. LPS non-significantly increased NR4A2 expression in both healthy control and COPD PMN at 120 minutes (Panel **A**). At 4 hours, no significant difference in NR4A2 expression between healthy control and COPD PMN was detected (Panel **B**). Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. qPCR expression levels were normalised by using a GAPDH standard curve. Abbreviations: HC – healthy control.

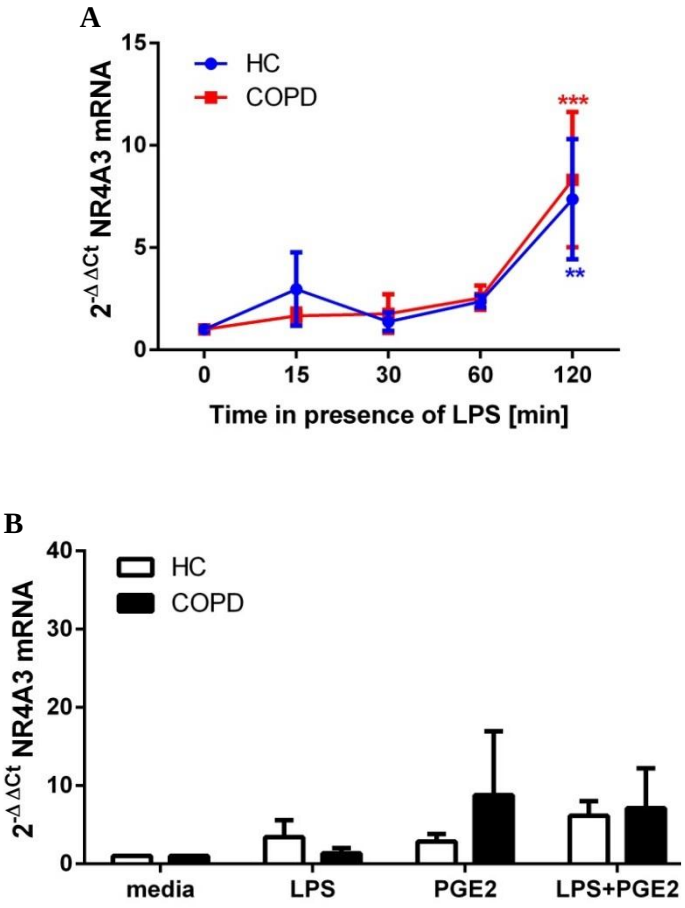


Figure 45: ***Effect of LPS and PGE2 on NR4A3 Expression in COPD and Healthy Control PMN.*** Percoll pure PMN from healthy control and COPD patients were incubated for 0 – 120 minutes with 10 ng/ml LPS (Panel **A**), or 4 hours with 10 ng/ml LPS, 10 μM PGE2, or both for 4 hours (Panel **B**). mRNA was extracted using the TRI method (as described in chapter 2.3.1.). Total cellular RNA was reverse transcribed into cDNA using random primers. Resulting cDNA was then analysed by qPCR using primers for NR4A3 and GAPDH as a normalisation control. LPS significantly increased NR4A3 expression in both healthy control and COPD PMN at 120 minutes (Panel **A**). At 4 hours, no significant differences between healthy control and COPD PMN were detected. Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. qPCR expression levels were normalised by using a GAPDH standard curve. Asterisks (*) denote differences to control conditions. Results were considered to be statistically significant for $p<0.01$ (**) and $p<0.001$ (***). Abbreviations: HC – healthy control.

similarly low level of constitutive PKA signalling in the PMN in this assay, as PKA phosphorylates, and thereby activates PDE4 (MacKenzie *et al.*, 2002). Therefore, the activation of PKA and their effect on PMN survival was further investigated. As selective PKA RI agonism, but not RII agonism was sufficient in the induction of cAMP-dependent PMN survival (Parvathenani *et al.*, 2015), PKA RI specific agonists and antagonists were used.

4.3.1.2. PKA Agonism Extends PMN Survival.

A comparatively high agonist dose of 50 μM was required to induce significant PMN survival with dbcAMP (**Fig. 28**), consistent with the studies by Rossi *et al.* (1995) and Usher *et al.* (2002), where 2 mM or 100 μM dbcAMP induced PMN survival at 20 hours or 5 hours, respectively. The highest inhibition of PMN apoptosis by dbcAMP was well below the levels achieved for the agonist pair N6/8-AHA at 50 μM and 100 μM (**Fig. 29B, C**). This may in part relate to their specific induction of PKA RI activity, as Type I specific agonist pairs previously induced survival, in contrast to Type II specific pairs (Parvathenani *et al.*, 1998). An explanation for the lower efficiency of dbcAMP at extending PMN survival might be the action of butyric acid (Stadl *et al.*, 1987; Graham *et al.*, 1988; Rivero *et al.*, 1998; Cuisset *et al.*, 1998; Sjöholm *et al.*, 1997; Conway *et al.*, 1998, Salminen *et al.*, 1998; Soldatenkov *et al.*, 1998). The activity of dbcAMP towards PKA is dependent on the enzymatic action of esterases, which catalyse the reaction of dbcAMP to monobutyryl cAMP and butyric acid (BTA). *In vitro*, the presence of esterases in the serum can allow this reaction to already occur in the culture medium (Köckritz-Blickwede, Chow, Nizet, 2009). To minimise the variation introduced by serum, a single batch (Lot #07372381) of heat inactivated FCS was used in all assays. Nevertheless, heat inactivation was unable to inactivate some types of thermally stable esterases contained in serum (Köckritz-Blickwede, Chow, Nizet, 2009), which allows for the possibility that action of esterases might affect dbcAMP survival effects. The released reaction products, namely monobutyryl cAMP, monobutyryl cGMP, cAMP and BTA can then have their own biological effects, which include the stimulation of gene expression in proliferation and apoptotic death (Schwede *et al.*, 2000a). In this context, monobutyryl cAMP shows increased membrane permeability and stability towards PDE metabolism (Schwede *et al.*, 2000b), which might potentially increase the effect of the drug on PMN survival. However, BTA induced apoptosis in various cell types, including PBMC (Kurita-Ochiai, Fukushima, Ochiai, 1999), which might mask the survival effect mediated by dbcAMP or monobutyryl cAMP.

The active metabolite of dbcAMP is the PDE resistant cAMP analogue N6-MB-cAMP, which is highly effective in the distinction between PKA and Epac (Christensen 2003). However, cAMP and its analogues possess varying inherent binding affinities towards the two distinct cyclic nucleotide binding (CNB) domains on the regulatory PKA subunits (Christensen 2003). As previously elaborated, a requirement for the activation of PKA is the occupation of both CNB

domains. Hence, in this chapter, the synergistic pair N6-MB-cAMP and 8-AHA-cAMP with affinities towards the CNB domain sites A and B (**Table 7**; Christensen *et al.*, 2003) were employed to synergistically activate PKA, as there is a lack of other 8-modified cAMP analogues with increased selectivity towards PKA over Epac.

The effect on PMN survival induced by BTA was further defined by using N6/8-AHA, both of which are insensitive to esterase degradation (Christensen *et al.*, 2003; Kopperud *et al.*, 2003), due to their chemical structures. Moreover, both, N6-MB-cAMP and 8-AHA-cAMP are described by the manufacturer as possessing increased membrane permeability and stability towards PDE metabolism, whereas dbcAMP only possess a low, non-selective affinity towards PDE (Tilley al., 2002). Taken together, this is consistent with their increased efficacy at inducing PMN survival and the low pro-survival action of rolipram.

It has been suggested that cAMP possesses a greater affinity for RI subunits than RII subunits (RIIβ<RIIα<RIα<RIβ; Edelman, 1987; Taylor *et al.*, 1992; Pink, Dell'Acqua, 2003), and N6-MB-cAMP and 8-AHA-cAMP preferentially target PKA subunit I (**Table 7**). Previously, it has been suggested that the differential binding affinities of cAMP to the distinct PKA subunits may also mediate transient effects at low cAMP concentrations via RI subunits, whereas persistent effects of high cAMP concentrations may be more dominantly transmitted through the involvement of the RII subunits (Zippel, Baldassa, Sturani, 2004). This might indicate that the distinct regulatory PKA subunits may be involved in the sequential orchestration of the effects of cAMP at high or low agonist concentrations. Interestingly, this may be consistent with the finding that RIα has been shown to be regulated by the increased expression other PKA subunits and showed additional compensatory functions upon loss of other regulatory subunits (Amieux *et al.*, 1997; Brandon *et al.*, 1997), supporting a more complex interplay of PKA subunits in the regulation of PMN survival, which would need to be addressed in an additional study.

4.3.1.3. PKA Antagonism increases PMN Apoptosis.

In a previous publication, the Sabroe/Whyte research groups showed that PMN survival induced by 1 mM N6-MB-cAMP can be inhibited by 0.7 mM of the competitive PKA Type I inhibitor Rp-8-Br-cAMPS at a late timepoint in Percoll pure PMN (Vaughan *et al.*, 2007).

The mechanism through which Rp-8-Br-cAMPS inhibits PKA, is by targeting the regulatory subunits RIα, RIβ and RIIα and competing for binding to the substrate sites with cAMP (Christensen *et al.*, 2003). Rp-8-Br-cAMPS preferentially targets the bound PKA RI subunits, but not the isolated RI subunits, which is thought to promote the stabilisation of the PKA holoenzyme with increased efficiency than 8-Br-cAMP (Gjersten *et al.*, 1995). Due to its chemical composition and structure, Rp-8-Br-cAMPS is also more lipophilic, and consequently, more membrane permeable than Rp-cAMPS (Gjertsen *et al.*, 1995), and shows increased resistance to PDE metabolism (Gjersten *et al.*, 1995; Butt, 2015). Moreover, Rp-8-Br-cAMPS

has a lower affinity towards Epac than comparable other PKA antagonists. This was demonstrated by Krakstad *et al.* (2004), who showed that the effect of the Epac negative control N6-MB-cAMP was completely blocked by Rp-8-Br-cAMPS (Krakstad *et al.*, 2004). Hence, Rp-8-Br-cAMPS would be thought to highly efficiently inhibit PKA signalling.

The relatively high levels of Rp-8-Br-cAMPS required to affect PMN survival are not surprising, considering that 8-Br-cAMP similarly displayed a low affinity towards PKA (Gjertsen *et al.*, 1995). However, the chosen concentration of 0.7 mM Rp-8-Br-cAMPS has previously been reported to induce a complete reversal of N6-MB-cAMP induced PMN survival (Krakstad *et al.*, 2004), demonstrating the value of Rp-8-Br-cAMPS in the investigation of PKA dependent PMN death. Moreover, no general cytotoxicity of 0.7 mM of the compound was observed by Krakstad *et al.* (2004), further highlighting the biological significance of the obtained results. This led us to conclude that the results obtained with Rp-8-Br-cAMPS are valid and biologically significant.

In a previous study, Rp-8-Br-cAMPS treatment did not influence constitutive survival of Percoll pure PMN at 16 hours (Vaughan *et al.*, 2007), suggesting that PKA signalling can be readily engaged, but is not constitutively active at a late timepoint following the separation from the blood. In addition, it was found here that 0.7 mM Rp-8-Br-cAMPS moderately increased constitutive PMN apoptosis at an early timepoint (**Fig. 30**), suggesting a transient survival effect induced by PKA signalling in PMN. Nevertheless, Rp-8-Br-cAMPS was only moderately efficacious at retarding constitutive PMN survival in one half of the donations (mean fold difference 4.6 % PMN apoptosis ± 1.2 SEM), while it profoundly increased apoptosis by a mean of 22.2 % (± 2.6 SEM) in the other moiety (**Fig. 6**). This difference was not correlated with the level of constitutive apoptosis in PMN, but might be grounded on constitutive differences in the basal cAMP levels. Various factors are able to influence intracellular cAMP levels (as previously discussed in chapter 1). Most relevantly, caffeine was shown to inhibit PKA signalling by blocking A2A receptors (Abo-Salem *et al.*, 2004). Insulin lowered cAMP levels, which was inhibited by glucose, which in turn increased cAMP production and activated PKA signalling in pancreatic endocrine cells (Elliott *et al.*, 2015). Thus, the difference between low responsive and high responsive PMN might be grounded in exogenous factors, such as natural variation induced by changes in the nutritional status (Oliveira *et al.*, 2014b; Elliott *et al.*, 2015; Zhao, Bruemmer, 2010), coffee consumption (Bassini-Cameron *et al.*, 2007), or alterations in culture conditions (i.e. purity of PMN preparation, variations in culture density; Sabroe *et al.*, 2004; Hannah *et al.*, 1998), whereas the difference is unlikely to be due to an inherent donor-dependent variable with genetic origin. Interestingly, this is supported by the reported dependence of the PKA signalling mediator subfamily NR4A on physiological cues, such as nutritional status and age (Pérez-Sieira *et al.*, 2014), and their induction by environmental stimuli (Zhao, Bruemmer, 2010; Martorell *et al.*, 2006). In summary, it was concluded that PKA

signalling can be readily engaged in PMN and is constitutively active at a lower level in most subjects.

4.3.2. The effect of autocrine and paracrine signals on PKA dependent neutrophil survival.

4.3.2.1. Low Levels of PBMC Extend PMN Survival.

Here, the influence of cell purity on constitutive PMN survival and PKA signalling was evaluated. Adding a small percentage (2 %) of PBMC into neutrophil cultures non-significantly delayed neutrophil survival at an early timepoint. Interestingly, Rp-8-Br-cAMPS induced a significantly lower amount of PMN apoptosis in the presence of PBMC. This indicates that the presence of PBMC might affect PKA survival signalling in PMN to a low extent, potentially through the secretion of pro-survival factors, as suggested by Sabroe *et al.* (2001). This underlines the importance of negative magnetic selection to obtain highly pure PMN in the investigation of PMN survival, as evidenced by Sabroe *et al.* (2004), and thereby reducing the variability in constitutive PKA dependence and constitutive levels of PMN survival (**Fig. 30**).

4.3.2.2. Autocrine Adenosine Extends PMN Survival Through PMN Survival.

The influence of a putative autocrine signal was examined, which has been suggested to be altered by fluctuations in cell culture density (Hannah *et al.*, 1998). In a supernatant transfer assay, PMN supernatant treatment was unable to rescue PMN survival at 20 hours, which led the authors to conclude that PMN were unable to produce autocrine factors in sufficient amounts to affect their survival. In contrast here, in an adaptation of the supernatant transfer assay performed at 4 hours PMN survival was prolonged by supernatant treatment (**Fig. 31B**), possibly suggesting that the autocrine factor is either shortlived or that the diminished growth factor content at 20 hours may have a detrimental effect on PMN lifespan, rendering PMN unable to respond to the autocrine signal.

This may well be reflected in the difference between the results of the two studies and further amplified by the ratio of supernatant to fresh media used. Here, we used supernatants in a 1:3 ratio with fresh media, while Hannah *et al.*, (1998) employed neat supernatants from a 20 hour culture, which thus encompasses the risk of depriving PMN of crucial survival factors. Moreover, Hannah *et al.* (1998) cultured neutrophils at a high density to obtain supernatants, while subsequently culturing them with a low PMN density. In contrast, here we used a constant density of PMN at a considerably shorter timepoint, which all may contribute to maintaining a minimal required level of serum constituents in culture.

Here, supernatant treatment increased PMN survival to a similar extent as PKA activation by dbcAMP at 4 hours (**Fig. 31B**), indicating that the survival factor might extend PMN survival through PKA activation. The PKA dependence and short-lived effects of the autocrine factor

may be consistent with the action of adenosine, which is rapidly taken up into the intracellular space due to the action of equilibrate nucleoside transporters and therefore often only exerts local effects by transient binding to the adenosine receptors A1, A2A, A2B, and A3 (Morote-Garcia *et al.*, 2013; Sitkovsky *et al.*, 2004). It was suggested that baseline adenosine signalling modulates constitutive survival in PMN (Yasui, *et al.*, 2000). Moreover, activated PMN were shown to release ATP, which is rapidly degraded to adenosine (Chen *et al.*, 2006), which mediates an autocrine feedback mechanism, regulating PMN function through A2 receptors (Chen *et al.*, 2004; Sitkovsky *et al.*, 2004). In this context, A2A receptors positively regulate PKA signalling through activation of AC (Pliyev *et al.*, 2014; Sitkovsky *et al.*, 2004), which sets them apart from other adenosine receptors.

To pursue the possibility that autocrine adenosine might enhance constitutive survival, the influence of adenosine and A2A receptors on PMN survival was examined. Increasing amounts of adenosine induced PKA-dependent PMN survival that was at least partially inhibited by A2A receptor antagonism (**Fig. 32A, B**), suggesting that adenosine induced PKA survival in PMN through the engagement of the A2A receptor. Moreover, antagonism of A2A receptors by ZM 241385 alone, increased constitutive PMN apoptosis; an effect that was mimicked by PKA inhibition through Rp-8-Br-cAMPS, indicating that PKA-dependent adenosine signalling is constitutively active through the involvement of A2A/PKA, consistent with the previous studies on the function of the receptor subtype (Pliyev *et al.*, 2014; Sitkovsky *et al.*, 2004).

This led us to conclude that the basal autocrine signal is governed by purinergic signalling in PMN. Interestingly, caffeine has previously been shown to interfere with adenosine signalling by blocking A2A receptors (Abo-Salem *et al.*, 2004). As caffeine levels following coffee consumption has been determined to be in the range of 10-50 μM in serum of healthy individuals (Baselt, 2011) and coffee consumption has previously been shown to influence neutrophil numbers (Bassini-Cameron *et al.*, 2007), the influence of caffeine on experimental procedures may often be underestimated. Thus, a donor dependent variable, influencing the assay might be dietary intake of caffeinated beverages of the donors prior to the blood donation. On the other hand, a previous study in monocytes did not detect an effect of caffeine on monocyte-induced PGE2 expression in response to LPS (Ulcar *et al.*, 2003) suggesting that if caffeine was to influence PMN survival, it would be unlikely to be an indirect PBMC-dependent mechanism.

Moreover, Insulin-induced reduction in cAMP levels was inhibited by glucose, which itself is a potent PKA activator in pancreatic endocrine cells (Elliott *et al.*, 2015). Thus, the difference between low responsive and high responsive PMN might also be grounded in exogenous factors, such as natural variation induced by changes in the nutritional status (Oliveira *et al.*, 2014b).

Taken together, these findings indicate that autocrine purinergic signalling constitutively activates cAMP/PKA signalling to a limited extend in PMN, but can be readily engaged, while the culture conditions and donor dependent variables might potentiate the influence of basal activity of PKA.

4.3.3. Mechanism of PKA-dependent Regulation of PGE2 Survival.

4.3.3.1. PGE2 Mediates PMN Survival via EP2.

PGE2/EP2 signalling is known to increase intracellular cAMP in PMN through the action of AC (Armstrong, 1995) and elevations in cAMP increase PMN survival (Rossi *et al.*, 1995). PDE4 is the main PDE subtype in PMN (Bäumer *et al.*, 2007). Therefore, it was hypothesised that PDE4 inhibition would enhance PGE2 induced survival by blocking cAMP degradation. In this study, rolipram alone showed little effect at concentrations up to 100 nM, and only slightly increased PMN survival thereafter (**Fig. 27**), indicating that there is a low constitutive activity of PDE4 and/or low constitutive cAMP levels present in PMN. In contrast to this, Armstrong (1995) found that 100 nM rolipram was sufficient to inhibit PMN chemotaxis in fMLP-stimulated Percoll-pure PMN (84 % purity). The higher efficacy of the reagent in the study by Armstrong (1995) may be grounded in the influence of PBMC on PKA-dependent PMN survival. In support, it was shown here that ultrapure PMN had 4 fold higher levels of constitutive apoptosis than Percoll pure PMN (**Fig. 36**), and the pro-apoptotic effect of PKA-inhibition by Rp-8-Br-cAMPS was an increase of 1.9 fold in ultrapure PMN and 4 fold in Percoll pure PMN, suggesting that the pro-survival role of PBMC may be exerted through the secretion of PKA-activating pro-survival factors.

Therefore, the role of PDE4 inhibition upon PGE2-mediated engagement of PKA signalling was investigated, to determine whether rolipram might potentiate the effect of the PKA activator. PGE2 was therefore incubated with PMN, at a concentration range that previously induced PMN survival (**Fig. 7**). The chosen concentrations of rolipram were based upon a study, where rolipram altered functional responses in PMN at these concentrations (Talpain *et al.*, 1995; Armstrong, 1995). Constitutive PMN survival was not significantly enhanced by PDE4 inhibition with 50 – 100 nM rolipram (**Fig. 34A**). This is consistent with a low constitutive activity of PDE4 in PMN that is not further activated through cAMP stimulation. Interestingly, 1 μM PGE2 alone did not significantly increase PMN survival at 4 hours in this assay, whereas addition of 100 nM rolipram to the PGE2 sample significantly increased PMN survival (**Fig. 34B**), and consistent with Talpain *et al.* (1995) and Armstrong (1995), where the PGE2-induced inhibition of superoxide generation and chemotaxis was respectively enhanced by co-incubation with rolipram. These results may further support the low inherent activity of PDE4 in

highly pure PMN and consequently their low influence on PGE2 survival. However, these data do not shed light on the intracellular availability of basal cAMP stocks in ultrapure PMN.

To further substantiate the PKA dependence of PGE2-induced PMN survival, the PKA-inhibitor Rp-8-Br-cAMPS was used to block PGE2 survival. While Rp-8-Br-cAMPS constitutively increased apoptosis by only 6 % (**Fig. 35A**), PKA inhibition also shifted the dose response curve for PGE2 (**Fig. 35C**). These results are consistent with previous studies, which reported delays of PMN apoptosis by elevations in cAMP (Rossi *et al.*, 1995), by PGE2 treatment (Ottonello *et al.*, 1998) and upon treatment with PKA RI-specific agonist pairs (Parvathenani *et al.*, 1998). This is in contrast to the study by Martin *et al.* (2001), where the pro-survival effect of cAMP in PMN was suggested to be independent of PKA. In particular, they found that dbcAMP and PGE2 induced late PMN survival, whereby PMN survival induced by dbcAMP could not be blocked by PKA inhibition, from which it was concluded that cAMP does not mediate PKA survival in PMN. Unfortunately, it may not have been known at the time of publication that dbcAMP is close to inactive towards PKA, and needs to be metabolised to MB-cAMP, MB-cGMP and butyrate to show functional effects. The reaction product butyrate has been implicated in various cellular processes (Yusta *et al.*, 1988), including the induction of gene expression, apoptosis and the interference with PKA signalling through the induction of PKC (Stadl *et al.*, 1987; Graham *et al.*, 1988; Rivero *et al.*, 1998; Cuisset *et al.*, 1998; Sjöholm *et al.*, 1997; Conway *et al.*, 1998, Salminen *et al.*, 1998; Soldatenkov *et al.*, 1998). Butyrate was furthermore shown to target the G-protein coupled receptor GPR43 (Le Poul *et al.*, 2003), which in turn was implicated in apoptosis signalling by inducing caspase activity (Tang *et al.*, 2011). This makes it a crucial requirement to control for the effects of butyrate in the investigation of PKA survival. Thus, the inability to block dbcAMP-induced survival may have been in part derived from the action of butyrate on PMN survival. Here, we showed that Rp-8-Br-cAMPS was in fact able to block survival induced by the cAMP-elevating agents adenosine (**Fig. 32B**) and PGE2 (**Fig. 35A**), indicating that the induced survival is PKA-dependent at an early timepoint, consistent with previous studies (Rossi *et al.*, 1995; Ottonello *et al.*, 1998; Parvathenani *et al.*, 1998). Moreover, this fits well with the effect of dbcAMP and PGE2 on the rapid enhancement of constitutive PKA activity at 30 minutes of treatment (Martin *et al.*, 2001), which is more relevant to the early PMN survival investigated here, in contrast to investigation of late PKA-dependent survival by Martin *et al.* (2001). However, these findings may also indicate that PKA-dependent survival is only transiently active, and more relevant at early timepoints after exposure to cAMP-elevating agents, as supported by **Fig. 36**, where PGE2 was ineffective at inducing late PMN survival.

Rp-8-Br-cAMPS more efficiently blocked PGE2-induced survival at lower agonist concentrations. Due to the competitive nature of Rp-8-Br-cAMPS binding to PKA (Gjertsen *et al.*, 1995), higher PGE2 concentrations and thus increased amounts of synthesized intracellular

cAMP, might be able to displace the competitive inhibitor from the cAMP binding sites. This may reduce the efficacy of the antagonist to bind the holoenzyme and to prevent its dissociation. Thus, it was concluded that PGE2-induced survival is PKA-dependent, and the relative loss of efficiency for higher PGE2 concentrations is due to the antagonist specificities, inducing a shift in the dose-response curve for PGE2.

Moreover, in chapter 3, the effects of EP2 on survival were hypothesised to be mediated through the engagement of the EP2 receptor subtype. Therefore, it was here examined, whether survival specifically induced by EP2 agonism can be blocked by inhibition of PKA. Consistently with **Fig. 35A**, constitutive survival was PKA-dependent in this assay (**Fig. 35B**). Moreover, butaprost-induced survival was blocked by PKA inhibition (**Fig. 35D**), indicating that the PKA dependence of PGE2 survival may be at least partially mediated via the prostaglandin receptor subtype EP2. Nevertheless, it should be noted that the agonist butaprost has a 10x lower affinity towards EP2 than PGE2 (**Table 6**). Thus, the PKA-dependent effect of EP2 signalling induced by butaprost may be even greater than anticipated. A comparison of the extent of PKA dependence in PGE2 and butaprost treatment curves suggests that PGE2 survival at lower concentrations is most likely mainly mediated by EP2. Yet, a higher PKA-dependence and increased effect of PGE2 at higher agonist concentrations was observed, which is not consistent with the reported affinity profiles (**Table 6**). It is possible that PGE2 mediates further pro-survival effects at higher concentrations through the non-specific engagement of an additional prostaglandin receptor subtype, due to the limitations of the pharmaceuticals (i.e. low affinity, non-selectivity, competition for binding). However, the identity of this potential receptor subtype remains to be confirmed.

4.3.3.2. Cell Purity Influences PGE2 Signalling in PMN.

Previously, low levels of PBMC were shown to increase PMN survival (Sabroe *et al.*, 2002a; Sabroe *et al.*, 2003). Moreover, late PMN survival induced by LPS was dependent on the presence of monocytes (Sabroe *et al.*, 2002a). In continuation of chapter 4.1.2., it was aimed here to examine the influence of PBMC on PGE2 induced PKA survival.

In this assay, constitutive PMN apoptosis was increased in highly pure PMN, whereas cell purity did not influence the PKA-dependence of constitutive PMN survival, as Rp-8-Br-cAMPS increased apoptosis in both Percoll pure (**Fig. 36A**) and highly pure PMN (**Fig. 36B**). However, in this assay PGE2 did not induce a significant increase in PMN survival at 4 hours. Interestingly, PKA-dependence was lost at 20 hours in highly pure PMN, whereas, PKA-dependence was maintained in PGE2 treatment samples of Percoll pure PMN. Considering the low PDE4 activity in PMN (**Fig. 27**, **Fig. 34**), the loss of PGE2 induced survival at 20 hours in PMN might be due to PGE2 degradation or uptake, which may be

counteracted by monocyte-derived PGE2 in presence of PBMC. This finding is consistent with Sabroe *et al*. (2002a), where monocytes induced PKA-dependent survival in PMN.

4.3.3.3. Early LPS–Induced Survival in PMN is Independent of PKA.

Previously, the Sabroe/Whyte laboratories showed that LPS transiently increased PMN survival at early timepoints, while the presence of monocytes was required to sustain late PMN survival in response to LPS (Sabroe *et al.*, 2002a). LPS treatment induced PGE2 expression via a Cox2 related mechanism in macrophages (Amann *et al.*, 2001; Ikegami *et al.*, 2001; Hofer *et al.*, 2004), suggesting that LPS might sustain late PMN survival via PKA in presence of monocytes. Additionally, in chapter 3.2., it was demonstrated that LPS upregulated receptor expression for prostaglandin receptors EP2 and EP4 and may thus potentially increase the susceptibility of PMN to PGE2 signalling.

As previously demonstrated (**Fig. 36A, B**), and corroborating the findings of Sabroe *et al.* (2002a), PMN survival was greater in the presence of PBMC (**Fig. 37A, B**). Moreover, LPS was more effective at increasing PMN survival in Percoll pure PMN, whereas PKA dependence was decreased by LPS treatment in both cell types (**Fig. 37A, B**). This may indicate that early LPS signalling induces the cessation of PKA signalling regardless of immune cell presence. Furthermore, PGE2 induced survival was PKA-dependent in both highly pure and ultrapure PMN. Strikingly, PKA dependence of LPS signalling was much more decreased in presence of monocytes, indicating that LPS acts through different PKA-dependent and independent pathways consistent with (Regenhard *et al.*, 2001; Wall *et al.*, 2009; Díaz-Muñoz *et al.*, 2012; Sabroe *et al.*, 2005), or that LPS reduced PKA dependent signalling, potentially through an indirect feedback mechanism involving EP receptor upregulation, as reported by (Ikegami *et al.*, 2001) in macrophages. Therefore, it was concluded that the presence of immune cells had a significant effect on LPS signalling; however, PKA dependence was influenced by the type of PMN stimulation, rather than immune cell presence.

As LPS survival retained a residual level of PKA dependence (**Fig. 37**), the dose-dependent relationship of LPS and PKA-dependence was examined. LPS induced PMN survival, whereas the effect of PKA inhibition decreased with increasing LPS concentrations and was entirely abolished at 1-10 ng/ml LPS (**Fig. 38A**). This may indicate that LPS not only induces a PKA independent form of survival, but in fact also diverts constitutive PKA signalling pathways with increasing efficiency, correlated with the concentration of LPS. Incubation with LPS and increasing concentrations of PGE2 increased PMN survival (**Fig. 38B**). Interestingly, Rp-8-Br-cAMPS increased PMN apoptosis on a constitutive level, while the extent of Rp-8-Br-cAMPS induced apoptosis by increasing PGE2 concentrations was attenuated in presence of LPS. This indicates that LPS does not interfere with constitutive PKA signalling; however, may lead to the cessation of PGE2-induced PKA signalling, potentially through the

inhibition of a signalling component upstream of PKA. With increasing PGE2 concentrations, the LPS-induced abrogation of PKA-dependent survival was rescued by PGE2 (**Fig. 38D**). Therefore, LPS induced signalling may partially regulate PGE2-induced PKA survival, but LPS treatment is not sufficient to inhibit PGE2-induced survival.

Since LPS induced monocytic PGE2 expression through Cyclooxygenase 2 (Cox2; Amann *et al.*, 2001; Ikegami *et al.*, 2001; Hofer *et al.*, 2004), the greater effect of LPS on PMN survival observed in these assays, may partially be explained by the induction of the Cox2/PGE2 pathways by LPS (**Fig. 37A, B**). However, in highly pure PMN, high concentrations of LPS induced a PMN survival that was independent of PKA signalling (**Fig. 38A, C**), indicating that LPS can induce PKA survival by the engagement of an additional signalling pathway. Interestingly, PGE2 was shown to increase intracellular cAMP in murine peritoneal macrophages, irrespective of LPS treatment, (Ikegami *et al.*, 2001), indicating that LPS would not affect PKA signalling in this way. A potentially indirect effect of LPS on PGE2-induced PKA signalling may be mediated through LPS-induced upregulation of EP2 and EP4 receptors. Cox2-derived intracellular PGE2 has been proposed to be a significant source of PGE2 in leukocytes, such as macrophages (Amann *et al.*, 2001; Ikegami *et al.*, 2001; Hofer *et al.*, 2004; Profita *et al.*, 2010). Strikingly, cigarette smoke extract increased PGE2 and Cox2 levels in macrophages, and may therefore also be involved in the extension of neutrophil survival in COPD (Profita *et al.*, 2010). Cox-2 derived PGE2 may further exert its action through the engagement of functional, perinuclear EP2 and EP4 receptors (Bhattacharya *et al.*, 1999; Konger *et al.*, 2005). Thus, the occurrence of prostaglandin receptor shuffling and internalisation may be the subject of a follow-up study.

4.3.3.4. NR4A2 and NR4A3 are Downstream Regulators of PKA-Dependent PGE2 Survival.

The mechanisms of PKA survival induced by PGE2 was investigated, and it was hypothesised that NR4A2 and NR4A3 may be critical parts of this pathway based on the upregulation of these nuclear receptors by a PKA agonist in a microarray (unpublished data). It was shown here that treatment with dbcAMP significantly increases expression of *NR4A2* and *NR4A3* at both investigated timepoints (**Fig. 39B**), indicating that *NR4A2* and *NR4A3* expression is activated through elevations of cAMP, and consistent with the reported induction of these molecules by PKA (Mohan *et al.*, 2012). Moreover, it has been noted that *NR4A2* and *NR4A3* expression decreases following the PMN preparation (**Fig. 39A, B**). The highly increased levels of *NR4A2* and *NR4A3* expression following the PMN isolation from the blood might constitute a cellular stress-response, as previously reported (Helbling *et al.*, 2014; Mohan *et al.*, 2012) or might actually reflect the constitutive levels of PMN in the blood. However, treatment with 100 μM dbcAMP entirely blocked the temporal decrease in *NR4A3* expression following the cell preparation (**Fig. 39B**) and partially inhibited time-dependent *NR4A2* decline, suggesting that

NR4A2 and *NR4A3* expression may become decreased during the onset of cellular aging and engagement of apoptosis pathways. Interestingly, there is substantial evidence for a pro-survival role of *NR4A2* and *NR4A3* (Mullican *et al.*, 2007), potentially supporting this hypothesis.

In a separate assay, PMN were incubated with adenosine, which is a well-known activator of PKA signalling. As expected, adenosine increased the expression of both *NR4A2* and *NR4A3* at an early timepoint (**Fig. 40A, B**), suggesting that both nuclear receptors are implicating in Adenosine/PKA signalling, as previously shown in monocytes (Crean *et al.*, 2015). A2A receptor antagonism prevented adenosine-induced upregulation of *NR4A2*, but not *NR4A3*, suggesting that NR4A3 is not engaged through the classical pro-survival receptor A2A, in contrast to NR4A2, consistent with Crean *et al.* (2015). In both cases, PKA antagonism efficiently blocked nuclear receptor upregulation by adenosine. This indicates that *NR4A2* and *NR4A3* mRNA expression can be increased by agonists that mediate elevations of intracellular cAMP, and that the upregulation of *NR4A2* and *NR4A3* is mediated through the activation of PKA. In line with the previous experiment, the increased *NR4A2* expression by adenosine may suggest that the effectivity of dbcAMP may be decreased due to the agonist specifics (as outlined above).

PGE2 treatment potently induced the expression of both *NR4A2* (**Fig. 41A**) and *NR4A3* (**Fig. 41B**). *NR4A3* upregulation was significantly blocked by PKA inhibition, indicating that at least *NR4A3* is a downstream effector of PKA signalling. Moreover, the LPS-induced expression of *NR4A3* was partially inhibited by constitutive PKA signalling, indicated by the significantly increased expression of *NR4A3* upon Rp-8-Br-cAMPS treatment in presence of LPS (**Fig. 42C**), when comparing NR4A expression in response to stimulation by LPS only. This may be explained by an indirect effect of LPS on PKA signalling, such as prostaglandin receptor upregulation, or stimulation of Cox2-derived PGE2 expression (Amann *et al.*, 2001; Ikegami *et al.*, 2001; Hofer *et al.*, 2004). More strikingly, LPS and PGE2 displayed synergy in the induction of *NR4A2* and *NR4A3* expression, and Rp-8-Br-cAMPS treatment only partially blocked the receptor upregulation, stabilising the receptor expression at a level observed for PKA inhibition in LPS-treated PMN (**Fig. 42A, B**). This indicates that LPS and PGE2 cooperate in the expression of both nuclear receptors and that PKA may be able to block the pathway involved in the LPS-induced *NR4A2* and *NR4A3* expression. Only recently, the induction of NR4A receptors through an Adenosine/A2A pathway and its further enhancement by TLR4 stimulation was demonstrated (Crean *et al.*, 2015), indicating that both LPS and PGE2 can cooperatively induce NR4A expression. Here, it was added to this by showing that PKA signalling may regulate the LPS-induced NR4A upregulation. Interestingly, both PKA and LPS/TLR4/MAPK cascades can activate the cAMP-responsive element binding protein (CREB; Roberson *et al.*, 1999), whereby the control of MAPK signalling by PKA is a well described occurrence (Stork, Schmitt, 2002). CREB regulates gene transcription upstream of *NR4A2* and

NR4A3 (Mohan *et al.*, 2012), and thereby, NR4A2 and NR4A3 may be involved in the transcriptional regulation of congruent outcomes of PGE2 and LPS-initiated signalling, such as their pro-survival effect in PMN, or the observed synergy in the production of IL-6, IL-10 or G-CSF (Strassmann *et al.*, 1994; Yamane *et al.*, 2000; Sugimoto *et al.*, 2005). Most interestingly, Crean *et al.* (2015) showed that the role of NR4A2 in inflammation was through the limitation of the effects of NFκB, and the control of an excessive inflammatory response. These results are in line with the low level of PKA-dependent survival induced by LPS for PMN survival, and may suggest a specific involvement of the nuclear receptors in PMN survival. As both, LPS and PGE2 are involved in PKA signalling and *NR4A2* and *NR4A3* mRNA is induced by dbcAMP, both nuclear receptors may be linked to the mediation of PKA-dependent pro-survival effects in PMN. This is supported by their involvement in PKA/CREB signalling (Mohan *et al.*, 2012) and the roles of the highly homologous (Zhao, Bruemmer, 2009), and partially redundant receptor NR4A1 in cell survival and proliferation (Nomiyama *et al.*, 2006; Park *et al.*, 2013; Hanna *et al.*, 2011; Mohan *et al.* 2012).

In chapter 3.1.2., an EP2 mediated phenotype of PGE2 survival was proposed. Here, it was shown that *NR4A2* and *NR4A3* are upregulated by PGE2 and EP2 agonism, but not EP4 agonism (**Fig. 43A, B**), supporting the findings of chapter 3 and placing NR4A2 and NR4A3 as downstream components of PGE2/EP2/PKA signalling, with a potential involvement in PMN survival. Previous research suggested that NR4A receptors are upregulated through Adenosine/A2A signalling in macrophages (Crean *et al.*, 2015), which shows congruency in the LPS and PGE2 mediated cellular effects (as discussed above).

Ultimately, the involvement of nuclear receptors NR4A2 and NR4A3 in COPD patient PMN was examined, to substantiate the clinical relevance of these findings. *NR4A3* was significantly increased through LPS treatment at 2 hours in both healthy control and COPD PMN (**Fig. 44A**), and a similar, but non-significant tendency was observed for *NR4A2* expression (**Fig. 45A**). This correlates well with the reported function of PMN as effector cells in COPD that are not inherently altered, but predominantly engaged by the unique alterations in the inflammatory environment in COPD. Due to their localisation downstream of PKA and CREB (Mohan *et al.*, 2012) and their synergistic expression by LPS and PGE2, they may be promising novel therapeutic targets for the treatment of neutrophilia in COPD. Interestingly, NR4A receptor targeting has previously been proposed as a therapeutical strategy in cancer, inflammation and metabolic disease (Mohan *et al.*, 2012; Deutsch *et al.*, 2012; Hsu *et al.*, 2004; Zhao, Bruemmer, 2010). However, the development of NR4A specific pharmaceuticals is hindered by the lack of an active site (Roshan-Moniri *et al.*, 2014).

Conclusively, to evaluate the applicability of NR4A2 and NR4A3 as therapeutic targets in PGE2-induced PMN survival in COPD, it was aimed to determine the specific role of both nuclear receptors in PMN survival. Although the number of publications on NR4A receptor has

been on the rise for the last year years, a lack of well-established agonists (Roshan-Moniri *et al.*, 2014) hinders the detailed investigation of the specific role of these nuclear receptors in PMN survival. In particular, a pharmacological activator or inhibitor of NR4A3 would prove to be of immense value in the investigation of PGE2 survival in PMN, as *NR4A3* was highly upregulated by dbcAMP and PGE2 treatment. To further address the role of NR4A2 and NR4A3 in PMN survival, an additional neutrophil model was employed in chapter 5 to modulate *NR4A2* and *NR4A3* expression levels.

4.4. Summary

In summary, we suggest here that PKA is a major pro-survival mediator in cAMP-mediated PMN survival, supported by the increased PMN survival in presence of dbcAMP and N6/8-AHA (**Fig. 28-29**), as well as the inhibition of constitutive survival by the PKA inhibitor Rp-8-Br-cAMPS (**Fig. 30**). However, the extent of constitutive cAMP-dependent survival varies, supported by the data achieved with Rolipram and Rp-8-Br-cAMPS (**Fig. 27**; **Fig. 30**). Even though most cell preparations showed close to 0 % apoptosis at 0 hours, the amount of constitutive cell survival at 4 hours varies considerably. Therefore, potential influences on the rate of apoptosis were investigated; in particular, autocrine and paracrine influences were investigated. It was found that constitutive PMN apoptosis was delayed by a small degree through variations in PBMC presence, as well as autocrine adenosine, acting through A2A receptors (**Fig. 31-33**).

PGE2 is a pro-survival mediator in PMN, acting via the EP2 receptor subtype and through activation of PKA. This was shown in **Fig. 35**, where effects of PGE2 on survival were thought to be mediated by EP2 at low agonist concentrations. However, higher PGE2 concentrations showed an additive effect, which was not attributable to EP2, but potentially to the engagement of another unknown receptor or signalling pathway. Here, it was proposed that this receptor is unlikely to be the EP4 receptor.

In **Fig. 36**, it was confirmed that the presence of contaminating cells (i.e. Percoll pure PMN), enhances the PKA-dependent pro-survival response of PMN at a late timepoint. Moreover, increased susceptibility to LPS-induced survival was observed in Percoll pure PMN in contrast to ultrapure PMN. This coincided with an increased rate of PKA-dependence in highly pure PMN, suggesting that LPS-induced survival is largely independent of PKA, whereas the mean overall survival was greater in Percoll pure PMN. A dose-response of LPS for PKA inhibition further confirmed that LPS induces a PKA-independent form of survival, and moreover dose-dependently terminates constitutive PKA-dependent survival signalling (**Fig. 38A, C**). The effect of LPS on PKA signalling was lost upon simultaneous treatment with PGE2, suggesting that LPS does not inhibit PKA, but may be involved in the regulation of PKA signalling upstream of PKA through an indirect mechanism (**Fig. 38B, D**).

Moreover, these data suggest that NR4A2 and NR4A3 nuclear receptors are downstream effectors of PKA signalling, as they become expressed by dbcAMP (**Fig. 39**), adenosine (**Fig. 40**) and PGE2 (**Fig. 41-43**). Expression induced by both Adenosine and PGE2 can be blocked by PKA-inhibition (**Fig. 40**; **Fig. 41C**; **Fig. 42**). Additionally, the EP2 agonist Butaprost non-significantly increased the expression of both *NR4A2* and *NR4A3* (**Fig. 43**). Butaprost was less efficient than PGE2, which might be explained by the different relative receptor affinities of the agonists (**Table 6**). LPS induced the expression of and *NR4A3* (**Fig. 45A**), correlating with the increase of EP2 and EP4 receptor expression induced by LPS (**Fig. 20-21**; **Fig. 22-23**).

In summary, here a novel mechanism in LPS enhanced PGE2 survival was proposed. In addition, the PKA-dependent effects of PGE2 on PMN survival are exerted via the EP2 receptor subtype. However, due to the limitations of the available pharmaceuticals (i.e. low affinity, non-selectivity, competition for binding), a further investigation of the mechanisms of the role of PKA-dependent survival in PMN is warranted. Therefore, cell type specific knockdown of PKA signalling partners might be useful in determining the exact role of PKA in PGE2 signalling.

CHAPTER 5. RNA INTERFERENCE MODULATES NR4A EXPRESSION IN MNØ.

Neutrophils are notoriously short-lived and post-mitotic cells and therefore they are genetically intractable. This obstacle can be overcome by the use of neutrophil precursor cell lines, which allows the genetic modification and subsequent differentiation of the cells into modified neutrophils. This allows the detailed investigation of the role of survival pathway components in neutrophils. In 1979, Gallagher *et al.* described the HL60 cell line, which are neutrophil progenitors derived from an acute promyelocytic leukaemia patient. HL-60 cells may be unsuitable as a model for primary human PMN for various reasons. Firstly, genetic variations have been described to increase the susceptibility for the development of leukaemia, which might indicate that HL60 cells are inherently different from healthy control PMN. Secondly, defects in the (malignant) proliferation of promyelocytes and their differentiation to neutrophils are well described in leukaemia. Moreover, the high passage numbers of available HL60 cells introduces the risk that genomic instability and chromosomal irregularities, potentially further altering neutrophil responses. Differentiation of HL60 cells is achieved by foreign reagents with largely unknown molecular mechanisms, such as DMSO. Taken together, the source of the precursors, their high passage numbers and the mechanism of their differentiation therefore are major limitations for the comparison between HL60 derived neutrophils and primary human neutrophils from healthy volunteers. This limits the usefulness of HL60 cells in the investigation of neutrophil survival pathways.

More recently, a novel approach for the generation of large quantities of genetically modified neutrophils from knockout mice has been described (Wang *et al.*, 2006). This approach is based on the discovery that Class I Homeobox (Hox) Transcription Factors are highly expressed in $CD34^-$ Haematopoietic stem cells (HSC) and myeloid lineage committed progenitors (LCP). Progressing differentiation is marked by increasing CD34 expression, coinciding with downregulation of *Hox* gene expression (Lawrence *et al.*, 1997, Pineault *et al.*, 2002). Moreover, expression of Hoxb8 has also been shown to directly arrest the differentiation of myeloid progenitors (Fujino *et al.*, 2001, Knoepfler *et al.*, 2001), and thereby allows the expansion of myeloid progenitors via expression of Hox oncoproteins.

For this end, lineage-negative progenitors ($MacI^-$, $B220^-$, $Thy1.2^-$, lin^-) were derived from the bone marrow of BALB/c mice and prestimulated with SCF, IL-3 and IL-6 by Wang *et al.* (2006). Subsequently, progenitors were infected with a retrovirus of a MMLV-derived retroviral vector (MSCV) encoding an estrogen-dependent (ER-binding domain fusion) Hoxb8 transcription factor. Expression of conditional Hoxb8 thus resulted in the factor-dependent immortalisation of the transfected progenitors. Upon inactivation of the conditional Hoxb8 and supplementation with neutrophil lineage specific growth factors, murine common myeloid

progenitor line (mCMP) terminally differentiated into neutrophils (mNØ; Wang *et al.*, 2006; McDonald *et al.*, 2011) that resembled mature murine neutrophils.

A modification of the protocol described by Wang *et al.* (2006) was published by McDonald *et al.* (2011), allowing the direct modification of gene expression in the lineage committed progenitors. Thereby, mature mutant neutrophils were directly derived from knockout (KO) progenitors, circumventing the cost- and time-consuming step of generating KO mouse models. Therefore, in this chapter, I examined the suitability of mCMP to model primary neutrophil apoptosis and PKA survival pathways. Thereafter, the feasibility of RNAi mediated transient gene expression knockdown of the nuclear receptors NR4A2 and NR4A3 was evaluated, and it was explored whether modification of NR4A signalling alters cell survival during mNØ differentiation.

5.1. mNØ: A Model for the Exploration of PMN Survival.

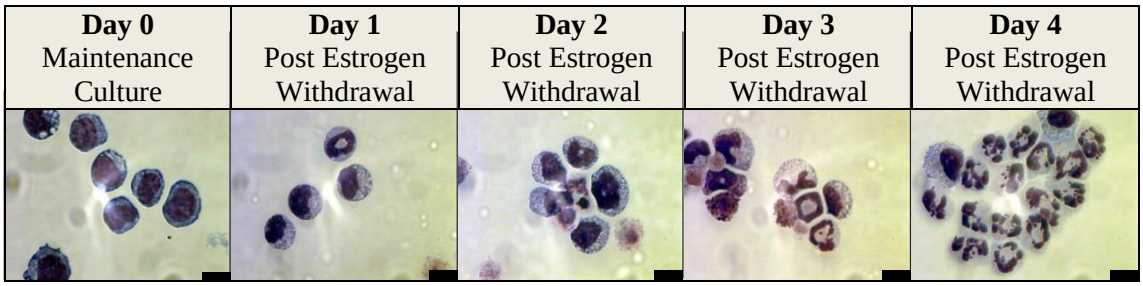
A conditionally immortalised mCMP (kind gift of Prof. P. R. Taylor) was cultured and differentiated into mature neutrophils (mNØ) in a 4-day protocol with daily media replenishment. The aim of this chapter was to assess mNØ apoptosis and survival to determine the applicability of the model for this study.

5.1.1 mCMP Differentiate into Highly Pure Populations of mNØ with High Morphological Resemblance to Murine Neutrophils.

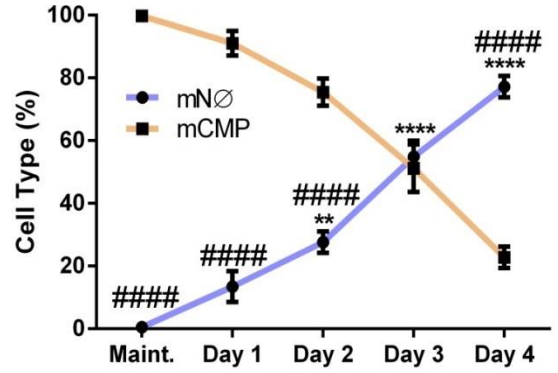
mCMP were maintained as conditionally immortalised progenitors in the presence of estrogen and SCF in long-term culture as described in chapter 2.1.1. Morphologically, mCMP exhibited a myeloid phenotype. Estrogen withdrawal resulted in increased cell death, but also enabled spontaneous differentiation of the mCMP into diverse cells, amongst which macrophages and eosinophils were most abundant at light microscopic observation (data not shown).

Supplementation with neutrophil-specific growth factors drives differentiation into morphological mature primary murine neutrophils (Wang *et al.*, 2006). Through estrogen withdrawal and supplementation with SCF and G-CSF, mCMP were differentiated into mature mNØ in a 4-day protocol (**Fig. 46A, B**). On day 2 of differentiation, the first significant increases in mNØ and decreases in mCMP were observed. The amount of differentiation into other cell types was negligibly low. On day 4, differentiation cultures routinely contained 20-40 x 10^6 of $\geq$ 95 % mNØ. On average, Day 4 cultures were composed of 80-90% mature lobulated mNØ (**Fig. 46C**) and 5-15% band-like mNØ, while the numbers of mCMP and intermediate differentiation stages was negligibly low.

A



B



C

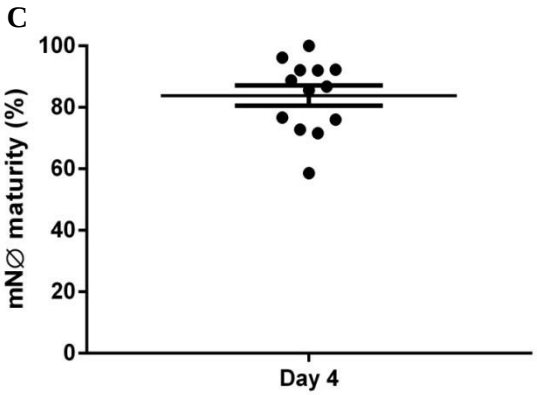


Figure 46: ***Differentiation of mCMP into Highly Pure, Morphologically Mature Neutrophil-like Cells (mNØ).*** mCMP were differentiated into mNØ in a 4 day timecourse, as described in 2.1.2. Apoptosis was quantified by light microscopy of Wright/Giemsa stained cells. Bars represent 10 µm (Panel **A**). Red line and blue line indicate percentage of mCMP or mNØ, respectively. Relative percentage of mNØ increased on days 0 – 4 post Estrogen withdrawal, with corresponding decreases in mCMP percentage (Panels **A**, **B**). Average purity of mature mNØ on day 4 post differentiation was above 80 % (Panel **C**). Cell maturity was assessed based on the presence of lobulated nuclei of mNØ (see **Fig. 4**). Data shown are mean ± SEM of 7 independent experiments (Panel **A**, **B**) or 13 independent experiments (Panel **C**) with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Statistical analysis was performed through Two Way ANOVA with Sidak's posttest (Panel **B**). Asterisks (*****) indicate significant differences to the maintenance control and octothorpes (#) denote differences between mCMP and mNØ in the differentiation culture. Results were considered to be statistically significant for $p<0.01$ (**) and $p<0.0001$ (****/####).

5.1.2. mNØ Lifespan Is Regulated Through Spontaneous Cell Death.

Like human PMN, murine neutrophils undergo apoptosis shortly following their release from the bone marrow *in vivo* (Pillay *et al.*, 2010) and during aging in culture. It was therefore examined whether mNØ displayed similarly high rates of spontaneous cell death. Spontaneous cell death was examined in media, supplemented with the full amount of growth factors. Additionally, growth factor deprived media was used to model the release of neutrophils from the bone marrow into the blood stream.

Morphologically mature mNØ populations were obtained on day 4 of differentiation cultures, and maintained in culture for a further 12 – 48 hours. Apoptosis was examined by two different measures. Firstly, relative abundance of cells with apoptotic morphology was measured by light microscopy, as described in chapter 2. Secondly, cell suspensions were stained with Annexin V-PE. Annexin V binds to cell surface phosphatidyl serine (PS), a receptor that is known to be redistributed to the outer membrane of apoptotic cells from the internal plasma membranes (Van Engeland *et al.*, 1998; Fadeel *et al.*, 1998; Fadok *et al.*, 2001; Hampton *et al.*, 2002). Additionally, cells were stained with the vital dye, Topro3, to measure necrotic like cell death (Van Hooijdonk *et al.*, 1994). Gating was based on both Topro3 and PE-annexin V staining. Position of gates was determined by a freeze/thaw control to model membrane permeability and the use of the calcium chelator EDTA to inhibit the calcium-dependent binding of Annexin V to PS. Furthermore, cell loss was measured via relative abundance of healthy cells remaining in culture compared to a predefined amount of flow cytometric beads. In growth factor deprived media, significantly increased levels of morphological apoptosis were first present after 36 hours (**Fig. 47A**, **C**). Likewise, the mean percentage of $FL2^{+}/FL4^{-}$ mNØ was significantly greater at 24 hours, compared to the 0 hours control (**Fig. 48A**, **D**).

Cell loss was determined by measurements of remaining viable cells through haemocytometer counts and CountBright beads. Viable cell numbers were significantly decreased after 12 hours measured by either haemocytometer counts (**Fig. 47B**) or CountBright beads (**Fig. 48**). Notably, only 25×10^4 viable cells were detected at the beginning of the timecourse through CountBright beads, compared to 50×10^4 viable cells by haemocytometer counts.

The growth factors SCF and G-CSF are implicated in neutrophil haematopoiesis and with known pro-survival functions in PMN (Hoffmann *et al.*, 1993; Duarte, Frank, 2002; Roberts, 2005; van Raam *et al.*, 2008). Since SCF and G-CSF are used in the differentiation of mCMP to mNØ, the influence of the growth factors on mNØ lifespan was investigated. mNØ were cultured in the presence and absence of SCF and G-CSF for 12 and 24 hours, before samples were collected for further analysis. In both growth factor deprived and supplemented media, mNØ exhibited increasing amounts of PS exposure in membrane impermeable cells ($FL2^{+}/FL4^{-}$; **Fig. 49A, D**). Likewise, the overall amount of viable cells remaining in the culture significantly decreased over time at a similar rate for both conditions (**Fig. 49B**). However, at 24 hours

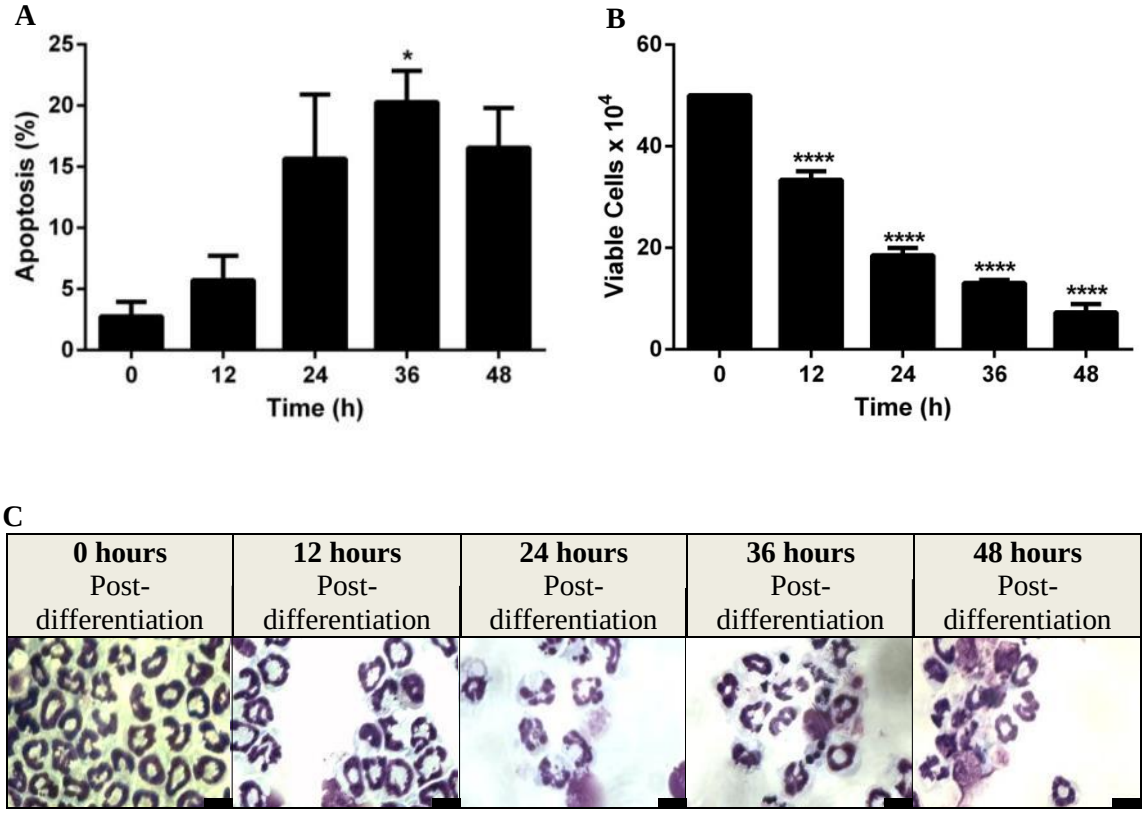


Figure 47: ***mNØ display Morphological Hallmarks of Apoptosis Constitutively and in a Time Dependent Manner.*** mNØ from a 4-day differentiation culture were seeded at 3.3 x 10^6/ml apoptosis medium, as described in chapter 2.1.2. mNØ were cultured for 0 – 48 hours before apoptosis was quantified by light microscopy of Wright/Giemsa stained cells (Panel **A**, **C**) and total cell numbers determined by Haemocytometer counts (Panel **B**). Relative percentage of morphologically apoptotic cells increased (Panel **A**, **C**) to a similar extent as means of viable cells decreased over time (Panel **B**). Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Cellular morphology was assessed by light microscopy. Bars represent 10 µm. Statistical analysis was performed through One Way RM ANOVA with Sidak's posttest. Results were considered to be statistically significant for $p<0.05$ (*) and $p<0.0001$ (****).

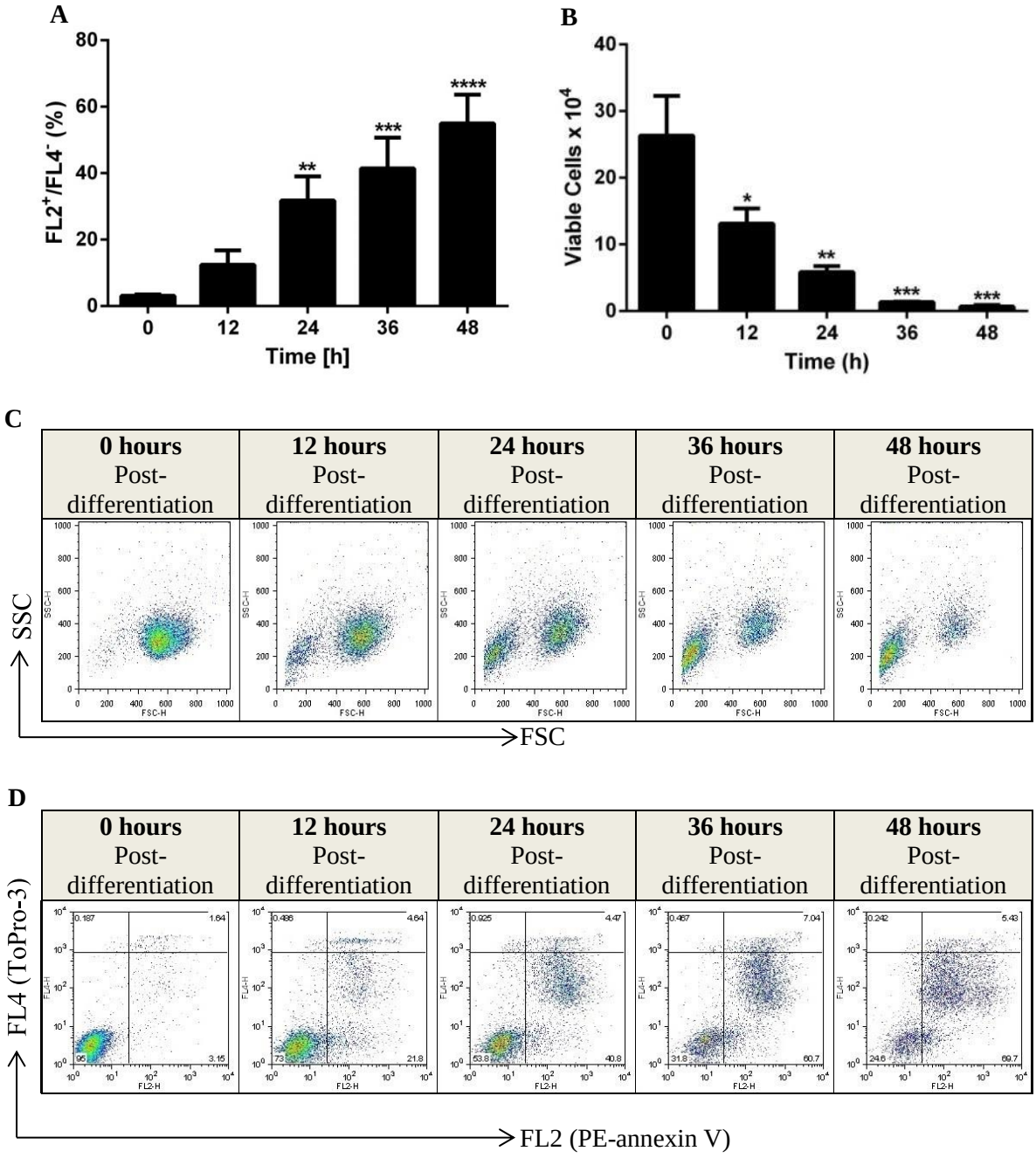


Figure 48: ***FACS Analysis of Time Dependent Phophatidylserine Exposure and Membrane Integrity in mNØ.*** mNØ from a 4-day differentiation culture were seeded at 3.3 x 10^6/ml apoptosis medium, as described in chapter 2.1.2. mNØ were cultured for 0 – 48 hours before apoptosis was quantified by FACS through PE-annexin V (FL2) and Topro-3 (FL4; Panels **A**, **D**) and total cell numbers determined through FACS analysis of CountBright beads (Panel **B**). Mean percentage of $FL2^+/FL4^-$ mNØ (Panel **A**) significantly increased over time, while means of viable cells/bead decreased (Panel **B**). Representative images show SSC/FSC graphs (Panel **C**) and FL4/FL2 dotplots (Panel **D**) of PE-annexin V/ToPro-3 stained mNØ at time points 0, 12, 24, 36 and 48 hours respectively. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Gates were established by EDTA, Freeze Thaw and unstained controls. Statistical analysis was performed through One Way ANOVA with Sidak's posttest. Results were considered to be statistically significant for $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p>0.0001$ (****).

significant differences between $FL2^+/FL4^-$ cells (< 10%) and viable cells (< 2 x 10^4) were observed between growth factor deprived and SCF/G-CSF treated cells.

5.1.3. mNØ Undergo Caspase-Dependent Apoptosis.

Apoptosis is a well-known mechanism of cell death in primary neutrophils. Moreover, there is considerable evidence to support the involvement of caspases in neutrophil apoptosis (Fadeel *et al.*, 1998). Q-VD-OPh (QVD) is a pan-caspase inhibitor, which has been shown to block apoptosis in human neutrophils (Wardle *et al.*, 2011). In contrast to other caspase inhibitors, Q-VD-OPh is highly effective, even at low concentrations. This was attributed to its high cell permeability and irreversible binding to activated caspases.

To investigate caspase-dependent apoptosis in this model system, morphologically mature mNØ populations were obtained on day 4 of differentiation cultures. Cells were cultured in presence and absence of Q-VD-OPh in full and growth factor deprived medium for 30 hours. Apoptosis was assessed through PE-annexin V/ToPro-3 staining and flow cytometric analysis. Cell loss was measured via relative abundance of viable cells remaining in culture compared to a constant amount of flow cytometric beads. Q-VD-OPh significantly reduced the levels of $FL2^+/FL4^-$ cells irrespective of growth factor presence (**Fig. 50A, D**). Q-VD-OPh and growth factor treatment both increased survival (**Fig. 50B, C**). Interestingly, cell survival induced by Q-VD-OPh treatment could not be further extended in the presence of growth factors.

5.2. PKA Survival Pathways Can Be Readily Engaged in mNØ.

The aim of this chapter was to assess the relevance of PKA signalling in the induction of mNØ survival, and thereby to determine their applicability as a model for the pathways in question.

5.2.1. mNØ Are Sensitive to Kinase Inhibition.

Staurosporine (STS) is a pan kinase inhibitor with a high affinity for the catalytic subtype β of PKA. Additionally to, or possibly as a consequence of its action on kinases, it has also been shown to activate caspase-dependent cell death (Belmokhtar *et al.*, 2001).

To determine, whether mNØ are sensitive to kinase inhibition, morphologically mature mNØ from a 4 day differentiation culture were cultured in the presence and absence of STS in full and growth factor deprived medium for 30 hours. STS modestly, yet significantly accelerated PMN apoptosis ($FL2^+/FL4^-$) at 30 hours compared to the untreated control (**Fig. 51A, C - D**). There was no significant loss of viable cells in presence of STS, but STS treatment resulted in a pronounced trend towards decreased viable cell numbers at 6 hours and 30 hours (**Fig. 51B**).

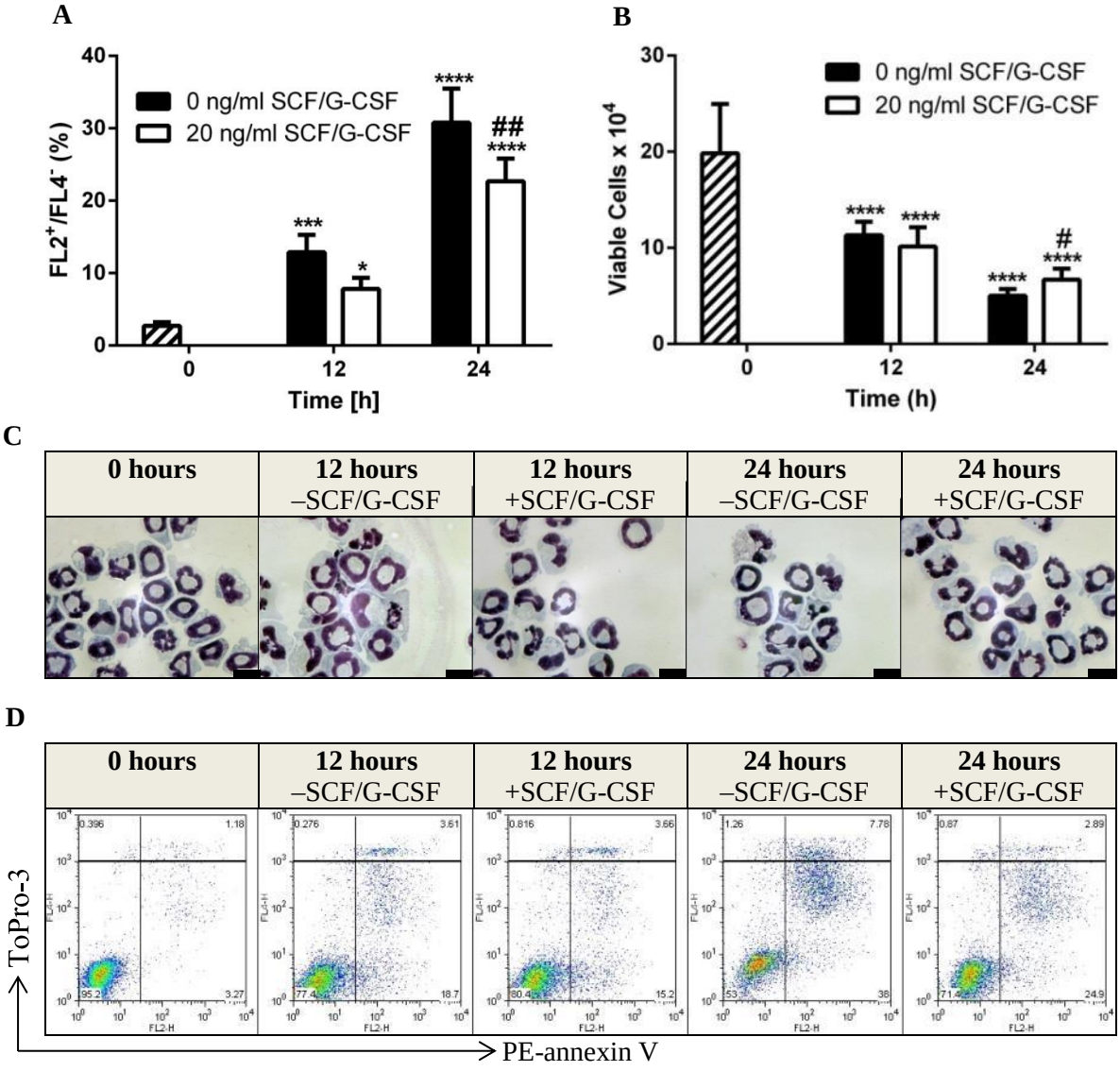


Figure 49: S*pontaneous mNØ Apoptosis is Accelerated through Growth Factor Deprivation.* mNØ from a 4-day differentiation culture were seeded at 3.3 x 10^6/ml apoptosis medium, as described in chapter 2.1.2. mNØ were cultured for 0 – 48 hours with or without 20 ng/ml SCF/G-CSF before apoptosis was quantified by FACS through PE-annexin V (FL2) and Topro-3 (FL4; Panels **A**, **D**). Total cell numbers were determined through FACS analysis of CountBright beads (Panel **B**). Mean percentage of $FL2^+/FL4^-$ mNØ (Panel **A**) significantly increased through growth factor deprivation, while retaining constitutive levels of apoptosis (Panel **A**). Means of viable cells/bead likewise increased at late timepoints in presence of growth factors (Panel **B**). Representative images show cellular morphology of mNØ (Panel **C**) and FL-4/FL-2 graphs of PE-annexin V/ToPro-3 stained mNØ (Panel **D**) at time points 12 hours and 24 hours, cultured with and without growth factors respectively. Data shown are mean ± SEM of 5 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Gates were established by EDTA, Freeze Thaw and unstained controls. Bars represent 10 µm. Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttest. Asterisks (*) represent differences to the 0 hour control, while octothorpes (#) denote differences between growth factor treatment conditions. Results were considered to be statistically significant for $p<0.05$ (*/#), $p<0.01$ (##), $p<0.001$ (***) and $p<0.0001$ (****).

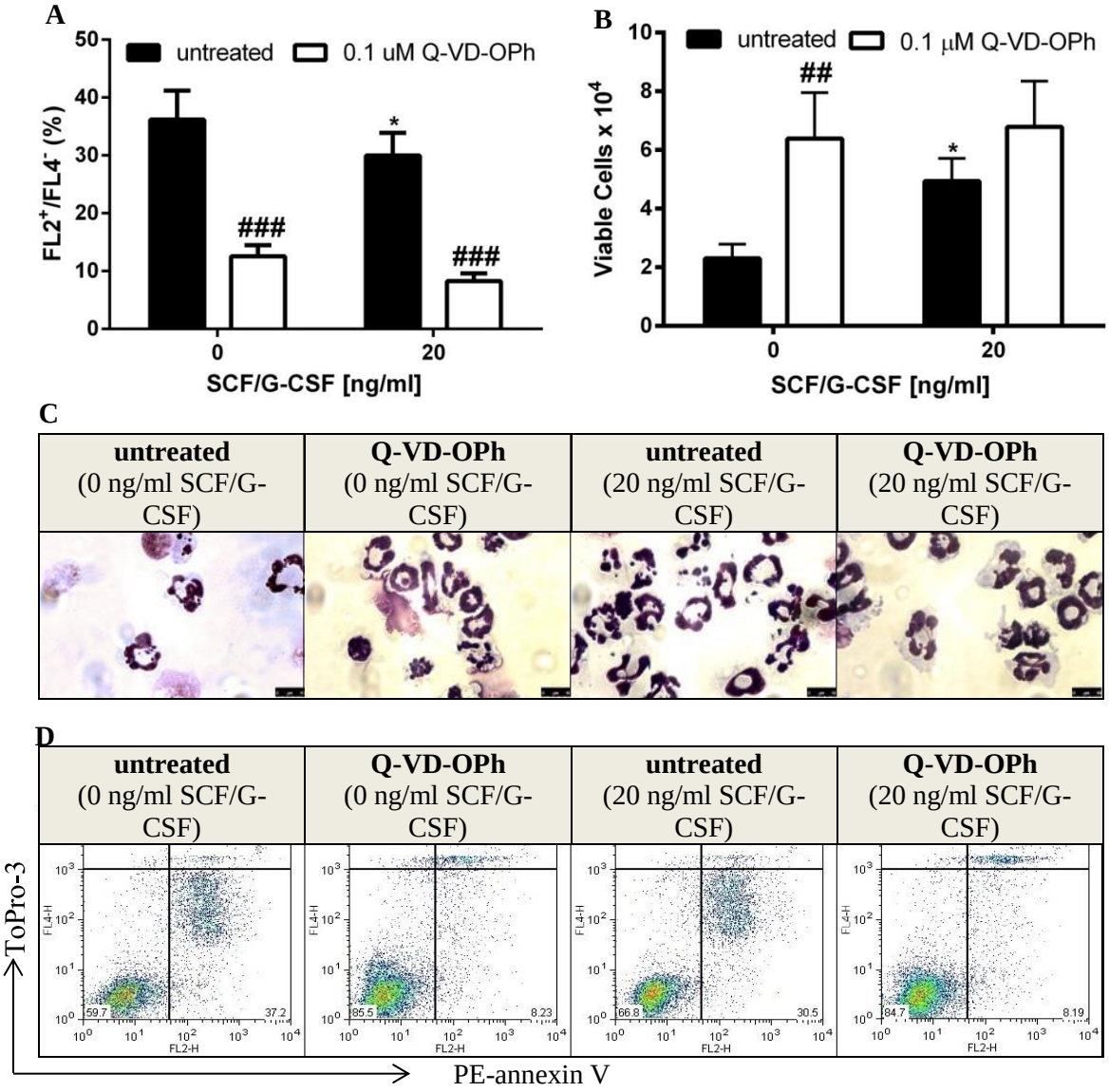


Figure 50: ***Evidence for Inhibition of Apoptosis by Caspase Inhibitor Q-VD-OPh in mNØ.*** mNØ from a 4-day differentiation culture were seeded at 3.3 x 10^6/ml apoptosis medium, as described in chapter 2.1.2. mNØ were cultured cultured in presence and absence of the Pan-Caspase Inhibitor Q-VD-OPh (0.1 µM) and with or without addition of 20 ng/ml SCF/G-CSF. mNØ were maintained for 30 hours before apoptosis was quantified by FACS through PE-annexin V (FL2) and Topro-3 (FL4; Panel **A - D**). Total cell numbers were determined through FACS analysis of CountBright beads (Panel **B**). Mean percentage of $FL2^+/FL4^-$ mNØ (Panel **A**) significantly decreased through treatment with 0.1 µM Q-VD-OPh independent of growth factor presence (Panel **A**). Relative abundance of viable cells compared to Countbright Beads similarly decreased in presence of Q-VD-OPh in starvation and fully supplemented media (Panel **B**). Representative images show cellular morphology of mNØ (Panel **C**) and FL-4/FL-2 graphs of PE-annexin V/ToPro-3 stained mNØ (Panel **D**) at 30 hours, cultured in presence or absence of QVD with or without growth factors. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Gates were established by EDTA, Freeze Thaw and unstained controls. Bars represent 10 µm. Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttest. Asterisks (*) mark differences to an untreated control, while octothorpes (#) denote differences to Q-VD-OPh treatment conditions. Results were considered to be statistically significant for $p<0.05$ (*), $p<0.01$ (##) and $p<0.001$ (###).

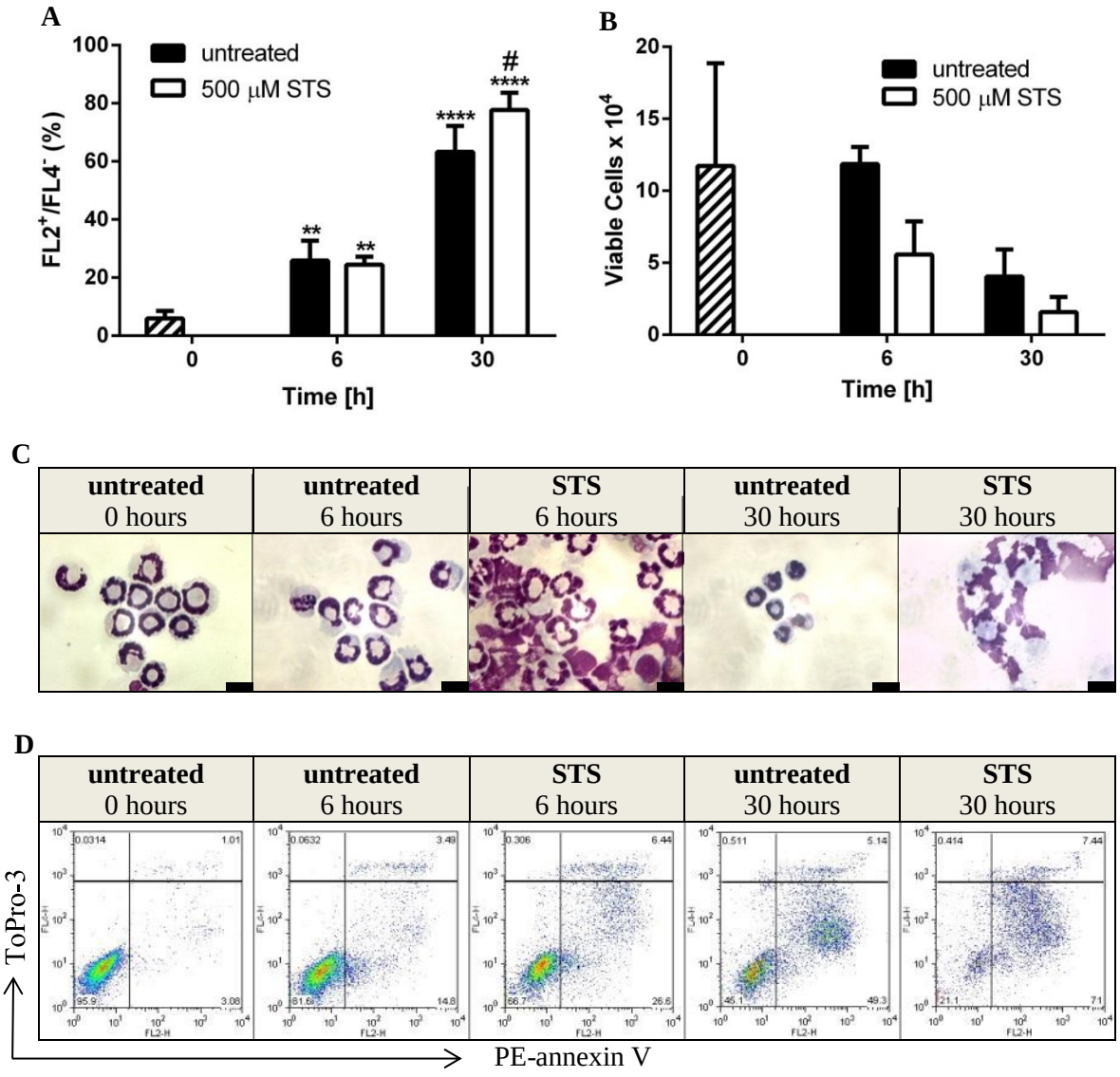


Figure 51: ***mNØ Apoptosis is Accelerated by Treatment with the Broad Spectrum Kinase Inhibitor Staurosporine.*** mNØ from a 4-day differentiation culture were seeded at 3.3 x 10^6/ml apoptosis medium, as described in chapter 2.1.2. mNØ were cultured in presence and absence of the broad spectrum kinase inhibitor STS (500 µM) for 6 and 30 hours before apoptosis was quantified by FACS through PE-annexin V (FL2) and Topro-3 (FL4; Panels **A, B, D**). Total cell numbers were determined through FACS analysis of CountBright beads (Panel **B**). Mean percentage of $FL2^+/FL4^-$ mNØ (Panel **A**) significantly increased at late timepoints through treatment with 500 µM STS (Panel **A**). Relative abundance of viable cells compared to Countbright Beads increased in presence of STS at early timepoints (Panel **B**). Representative images show cellular morphology of mNØ (Panel **C**) and FL-4/FL-2 graphs of PE-annexin V/ToPro-3 stained mNØ (Panel **D**) at 0-30 hours, cultured in presence or absence of STS. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Gates were established by EDTA, Freeze Thaw and unstained controls. Bars represent 10 µm. Statistical analysis was performed through Two Way ANOVA with Sidak's posttest. Asterisks (*) indicate differences to 0 hour control, while octothorpes (#) denote differences between STS treatment. Results were considered to be statistically significant for $p<0.05$ (#), $p<0.01$ (**) and $p<0.0001$ (****).

5.2.2. PKA Survival Pathways Can Be Engaged in mNØ.

In chapter 4, PKA was found to be an important mediator of PMN survival. Here, the validity of mNØ as a model system in the study of PKA survival was examined. The PKA activator dbcAMP and the analogue pair N6-MB-cAMP/8-AHA-cAMP were employed as PKA activators. Briefly, mNØ from Day 4 differentiation cultures were seeded at a density of 0.5 x 10^6 cells/well (96-well plates) in apoptosis medium. Stimuli were inoculated at a final concentration of 100 μM for dbcAMP or N6/8-AHA. A temporal increase of $FL2^+/FL4^-$cells (**Fig. 52A, D; Fig. 53A, D**) was significantly attenuated at 30 hours by incubation with dbcAMP (**Fig. 52A, D**), but not N6/8-AHA (**Fig. 53A, D**). The mean percentage of $FL2^+/FL4^-$ fluorescent cells was reduced by 30.8 % with dbcAMP and by 17.3 % with N6/8-AHA at 30 hours. For both assays, the number of viable cells in culture was increased at 6 hours of agonist treatment (**Fig. 52B**, **Fig. 53B**). Increases were significant for N6/8-AHA treatment only. However, for both assays, the effect on cell numbers was lost at 30 hours.

To substantiate the involvement of PKA signalling in mNØ survival, mNØ were incubated for 24 hours with Rp-8-Br-cAMPS. At 24 hours of treatment with 0.7 mM Rp-8-Br-cAMPS the mean percentage of $FL2^+/FL4^-$ mNØ was modestly yet significantly increased by 2.9 % (**Fig. 54A**). There was no significant change in mean viable cell numbers in Rp-8-Br-cAMPS treated conditions (**Fig. 54B**). Similarly, Rp-8-Br-cAMPS was unable to block the dbcAMP-induced reduction in $FL2^+/FL4^-$ cells (**Fig. 55A**). Viable cell numbers were not significantly affected by dbcAMP and Rp-8-Br-cAMPS (**Fig. 55B**).

5.2.3. Engagement of PKA Survival Through Inflammatory Stimuli in mNØ.

The effect of LPS and PGE2 on mNØ apoptosis and survival was then investigated and Rp-8-Br-cAMPS was used to substantiate the differential PKA-dependence of the survival mediators in mNØ. Therefore, mNØ from a 4 day differentiation culture were incubated with LPS, PGE2 or both, in the presence or absence of Rp-8-Br-cAMPS. LPS and PGE2 alone did not result in significant changes to numbers of $FL2^+/FL4^-$ or viable cells (**Fig. 56A, B**). Co-incubation with LPS and PGE2 induced a statistically significant increase in viable cell numbers that was significantly blocked by PKA inhibition with Rp-8-Br-cAMPS (**Fig. 56B**).

5.3. Transient RNAi Mediated Knockdown in mCMP Alters Gene Expression Levels in mNØ.

In this chapter, the ability to genetically modify mCMP and the resulting mNØ by siRNA transfection techniques was examined. Here, mRNA expression of several pro- and anti-survival molecules was modulated to determine their involvement in the regulation of mNØ lifespan.

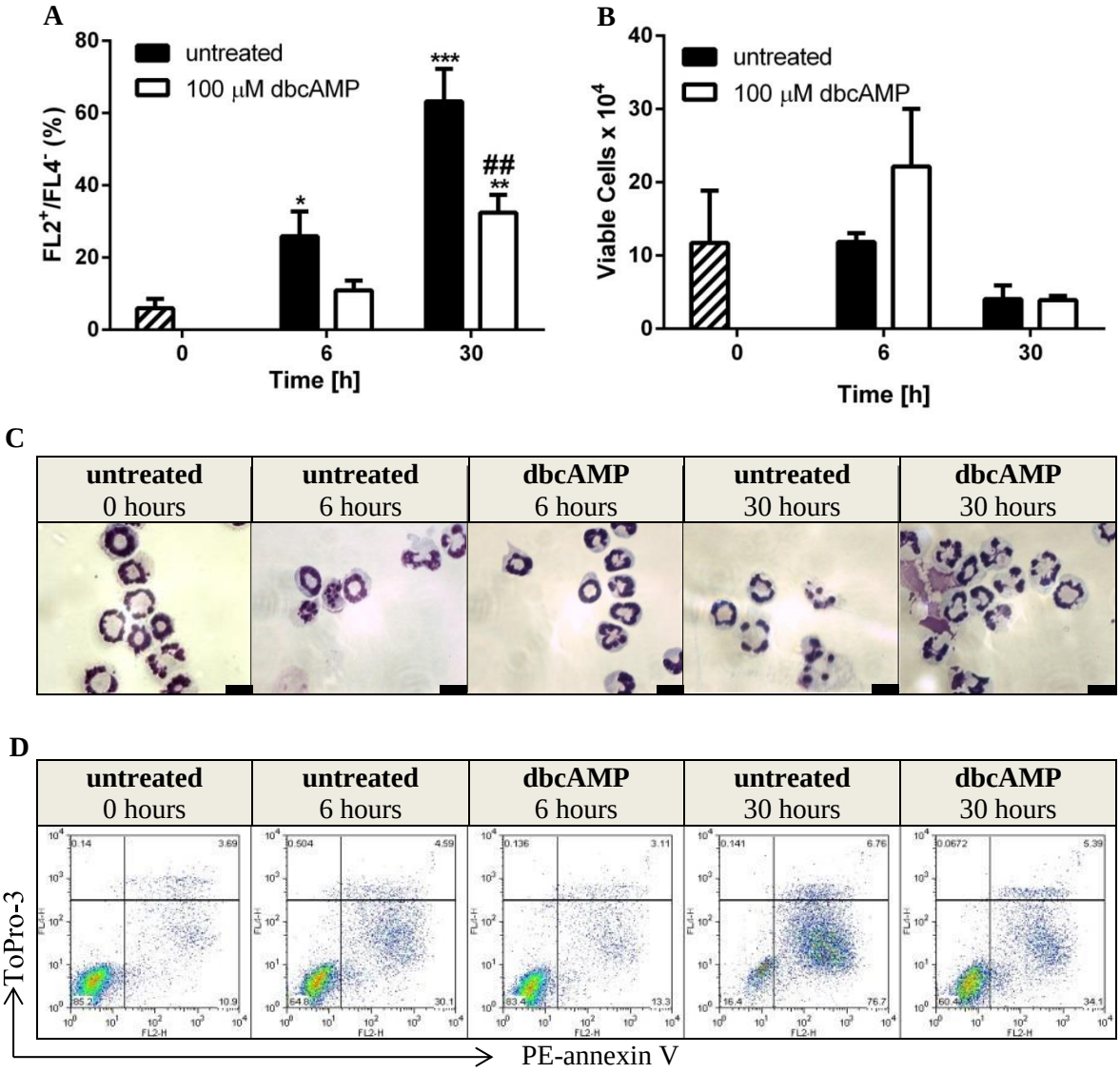


Figure 52: ***mNØ Survival is Activated by dbcAMP.*** mNØ from a 4-day differentiation culture were seeded at 3.3 x 10^6/ml apoptosis medium, as described in chapter 2.1.2. mNØ were cultured in presence and absence of 100 μM of the cAMP analogue dibutyryl cyclic AMP (dbcAMP) for 6 and 30 hours before apoptosis was quantified by FACS through PE-annexin V (FL2) and Topro-3 (FL4; Panel **A - D**). Total cell numbers were determined through FACS analysis of CountBright beads (Panel **B**). Mean percentage of $FL2^+/FL4^-$ mNØ was significantly lower for dbcAMP treated conditions (Panel **A**). Relative abundance of viable cells compared to Countbright Beads was enhanced in presence of dbcAMP (Panel **B**). Representative images show cellular morphology of mNØ (Panel **C**) and FL-4/FL-2 graphs of PE-annexin V/ToPro-3 stained mNØ (Panel **D**) of treatment conditions at 30 hours. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Gates were established by EDTA, Freeze Thaw and unstained controls. Bars represent 10 μm. Statistical analysis was performed through Two Way ANOVA with Sidak's posttest. Asterisks (*) indicate differences to 0 hour control, while octothorpes (#) denote differences between dbcAMP treatment. Results were considered to be statistically significant for $p<0.05$ (*), $p<0.01$ (**/##) and $p<0.001$ (***).

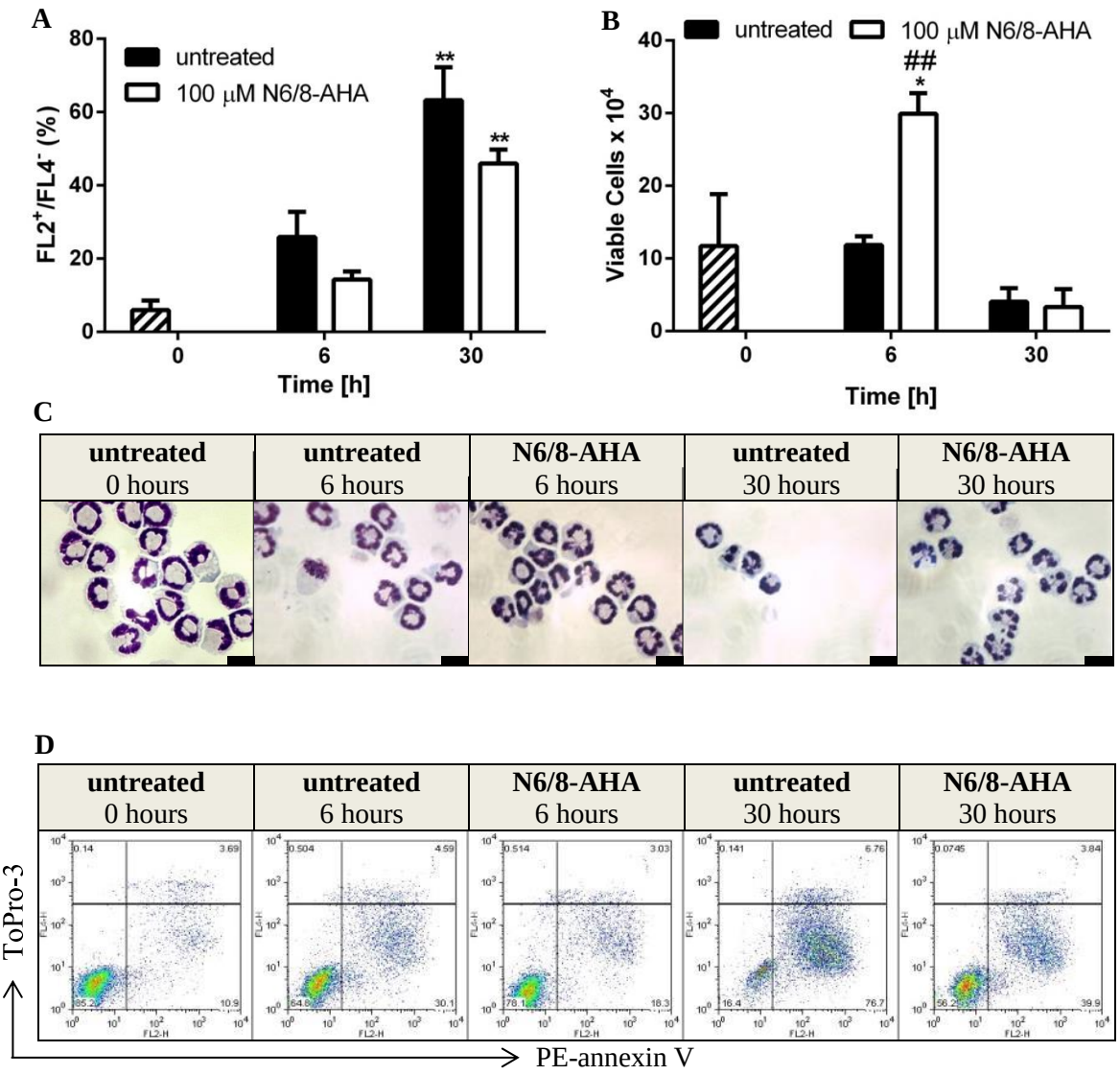


Figure 53: ***mNØ Survival is Activated by N6/8-AHA.*** mNØ from a 4-day differentiation culture were seeded at 3.3 x 10^6/ml apoptosis medium, as described in chapter 2.1.2. mNØ were cultured in presence and absence of 100 μM of the dbcAMP analogue-pair N6-MB-cAMP and 8-AHA-cAMP (N6/8-AHA) for 6 and 30 hours before apoptosis was quantified by FACS through PE-annexin V (FL2) and Topro-3 (FL4; Panel **A**, **D**). Total cell numbers were determined through FACS analysis of CountBright beads (Panel **B**). Mean percentage of $FL2^+/FL4^-$ mNØ was significantly lower for N6/8-AHA treated conditions at 30 hours (Panel **A**). Relative abundance of viable cells compared to Countbright Beads was significantly enhanced in presence of N6/8-AHA at 6 hours (Panel **B**). Representative images show cellular morphology of mNØ (Panel **C**) and FL-4/FL-2 graphs of PE-annexin V/ToPro-3 stained mNØ (Panel **D**) of treatment conditions at 30 hours. Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Gates were established by EDTA, Freeze Thaw and unstained controls. Bars represent 10 μm. Statistical analysis was performed through Two Way ANOVA with Sidak's posttest. Asterisks (*) indicate differences to 0 hour control, while octothorpes (#) denote differences between N6/8-AHA treatment. Results were considered to be statistically significant for $p<0.05$ (*) and $p<0.01$ (**/##).

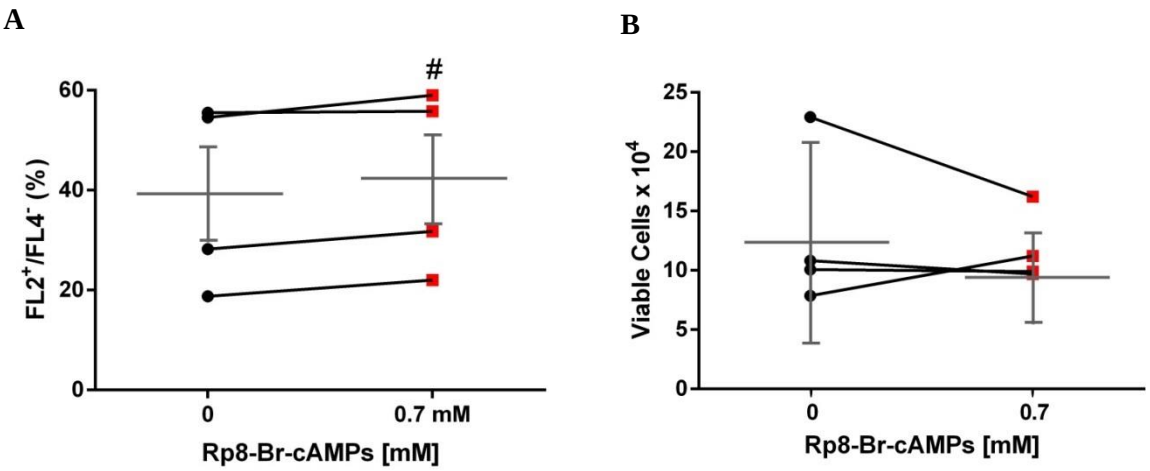


Figure 54: ***PKA Inhibitor Rp-8-Br-cAMPS Significantly Increases Constitutive mNØ Apoptosis***. mNØ from a 4-day differentiation culture were seeded at 3.3 x 10^6/ml apoptosis medium, as described in chapter 2.1.2. mNØ were cultured in presence and absence of 0.7 mM of the PKA antagonist Rp-8-Br-cAMPS for 24 hours before apoptosis was quantified by FACS through PE-annexin V (FL2) and Topro-3 (FL4; Panel **A**). Total cell numbers were determined through FACS analysis of CountBright beads (Panel **B**). Mean percentage of $FL2^+/FL4^-$ mNØ was significantly higher for Rp-8-Br-cAMPS treated conditions at 24 hours (Panel **A**). Relative abundance of viable cells compared to Countbright Beads was lower in presence of Rp-8-Br-cAMPS at 24 hours (Panel **B**). Data shown are single data points and mean ± SEM of 4 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Gates were established by EDTA, Freeze Thaw and unstained controls. Statistical analysis was performed through paired t tests. Significant differences between Rp-8-Br-cAMPS treated and control conditions were indicated by octothorpes (#). Results were considered to be statistically significant for $p<0.05$ (#).

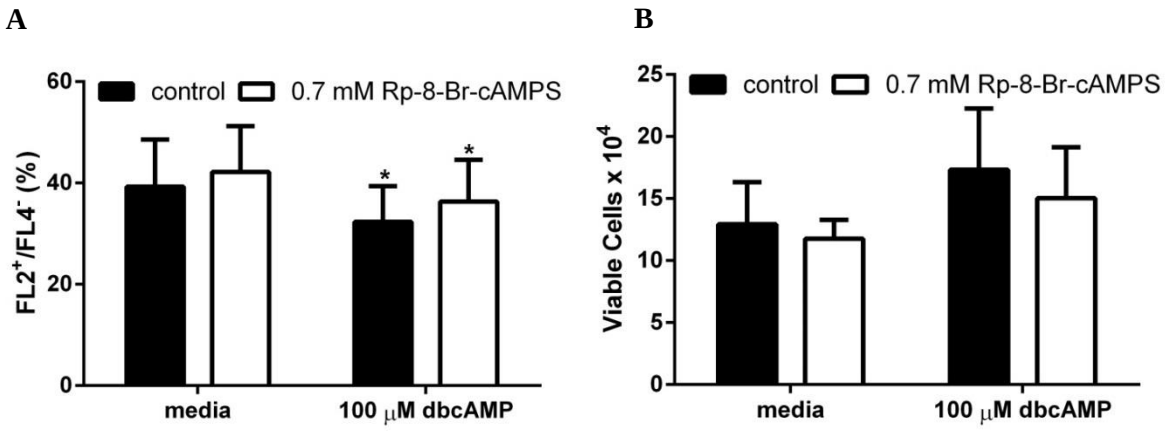


Figure 55: ***PKA Inhibitor Rp-8-Br-cAMPS does not Significantly Increase mNØ Apoptosis in Presence of the PKA Agonist dbcAMP.*** mNØ from a 4-day differentiation culture were seeded at 3.3 x 10^6/ml apoptosis medium, as described in chapter 2.1.2. mNØ were cultured in presence and absence of 100 μM of dbcAMP and 0.7 mM of the PKA inhibitor Rp-8-Br-cAMPS for 24 hours before apoptosis was quantified by FACS through PE-annexin V (FL2) and Topro-3 (FL4; Panel **A**). Total cell numbers were determined through FACS analysis of CountBright beads (Panel **B**). Mean percentage of $FL2^+/FL4^-$ mNØ was lower for dbcAMP treated conditions at 24 hours (Panel **A**). Relative abundance of viable cells compared to Countbright Beads was not significantly regulated by dbcAMP or Rp-8-Br-cAMPS treatment at 24 hours (Panel **B**). Data shown are mean ± SEM of 4 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Gates were established by EDTA, Freeze Thaw and unstained controls. Statistical analysis was performed through Two Way ANOVA with Sidak's posttest. Asterisks (*) indicate differences to the untreated control. Results were considered to be statistically significant for $p<0.05$ (*).

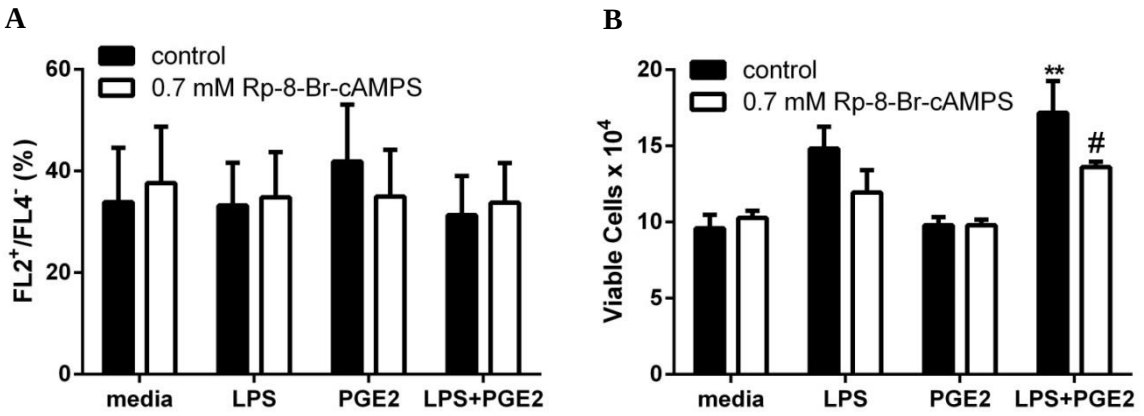


Figure 56: ***LPS and PGE2 Cooperate in the Induction of PKA-Dependent Prevention of Cell Loss in mNØ.*** mNØ from a 4-day differentiation culture were seeded at 3.3 x 10^6/ml apoptosis medium, as described in chapter 2.1.2. mNØ were preincubated for 15 minutes with 0.7 mM Rp-8-Br-cAMPS. Thereafter, mNØ were cultured in presence or absence of 1ng/ml LPS, 10 µM PGE2, or both for 30 hours before apoptosis was quantified by FACS through PE-annexin V (FL2) and Topro-3 (FL4; Panel **A**). Total cell numbers were determined through FACS analysis of CountBright beads (Panel **B**). Mean percentage of $FL2^+/FL4^-$ mNØ was significantly lower for treatment with both LPS and PGE2 (Panel **A**). Relative abundance of viable cells compared to Countbright Beads was significantly enhanced in presence of both LPS and PGE2, and treatment-induced increase in viable cells was blocked by Rp-8-Br-cAMPS-treatment (Panel **B**). Data shown are mean ± SEM of 3 independent experiments with cells isolated from distinct healthy volunteers, as described in chapter 2.5. Gates were established by EDTA, Freeze Thaw and unstained controls. Statistical analysis was performed through Two Way ANOVA with Sidak's posttest. Asterisks (*) indicate differences to control conditions, while octothorpes (#) denote differences in Rp-8-Br-cAMPS treatment conditions. Results were considered to be statistically significant for $p<0.05$ (#) and $p<0.01$ (**).

5.3.1. Optimisation of the RNAi Strategy.

Small interference RNAs (siRNA) were delivered into mCMP by nucleofection with an Amaxa Nucleofector, as previously described in chapter 2.3.6. To optimise the transfection efficiency, conditionally-immortalised mCMP were transfected with a non-targeting (siScrbl) and a CypB (siCypB) targeting siRNA pool and subsequently incubated for 48 to 96 hours. Transfection efficiency was assessed through the measurement of gene expression changes by RT PCR. *CypB* gene expression was reduced following transfection with *CypB* siRNA (**Fig. 57**). There was no change in gene expression at 24 hours post-transfection (data not shown). Gene knockdown was most efficient at 48 hours post-transfection (85 % compared to scrambled; **Fig. 57A, B**) and gene transcripts typically increased over time until 96 hours at which time only 12 % reduction in *CypB* mRNA was observed (**Fig. 57A, B**). Nucleofection reduced overall cell numbers (data not shown); however, numbers of viable cells recovered over the course of the next 72 hours (**Fig. 57C**). Moreover, there were no significant differences in viable cell numbers in siScbrl and siCypB transfected cultures (**Fig. 57C**).

Since transfection efficiency was sub-optimal at 96 hours posttransfection, siRNA transfection during the differentiation process of mCMP to mNØ is necessary to obtain newly matured neutrophils with the greatest gene knockdown. To optimise the transfection strategy, mCMP were transfected with an siRNA pool for siScrbl at D0 to D3 of differentiation (n=1). Transfection at D0 considerably reduced viable cell numbers during the differentiation which continued through to D4 posttransfection (**Fig. 58A**). Transfection at D1 of mCMP differentiation initially reduced viable cell numbers, while cell numbers recovered considerably over the following two days. Transfection on D2 on the other hand noticeably reduced cell numbers with a decreased recovery rate, and an overall reduced number of viable cells on D4. Transfection at D3 did not impair the recovery of cells and yielded the highest number of viable mNØ at D4 (**Fig. 58A**).

Transfection on D1 of the differentiation process was chosen as a suitable timepoint for mCMP transfection, due to acceptable numbers of viable cells (**Fig. 58A**), as well as gene knockdown efficiency at 72 hours post-transfection (**Fig. 57A, B**). Consequently, cells were transfected with the siRNA pools siScrbl and siCypB on D1 post estrogen withdrawal, and cultured in the presence of SCF and G-CSF for a further 3 days. There was no difference in viable cell numbers between siScrbl and siCypB transfected cultures (**Fig. 58B**). On D4, *mCypB* levels were reduced by 67.8 % (± 18.8 SEM) in siCypB transfected cells in comparison to the non-targeting control (**Fig. 58C, D**). This indicates that gene product knockdown is retained throughout the differentiation procedure by transfection of mCMP at D1 of differentiation.

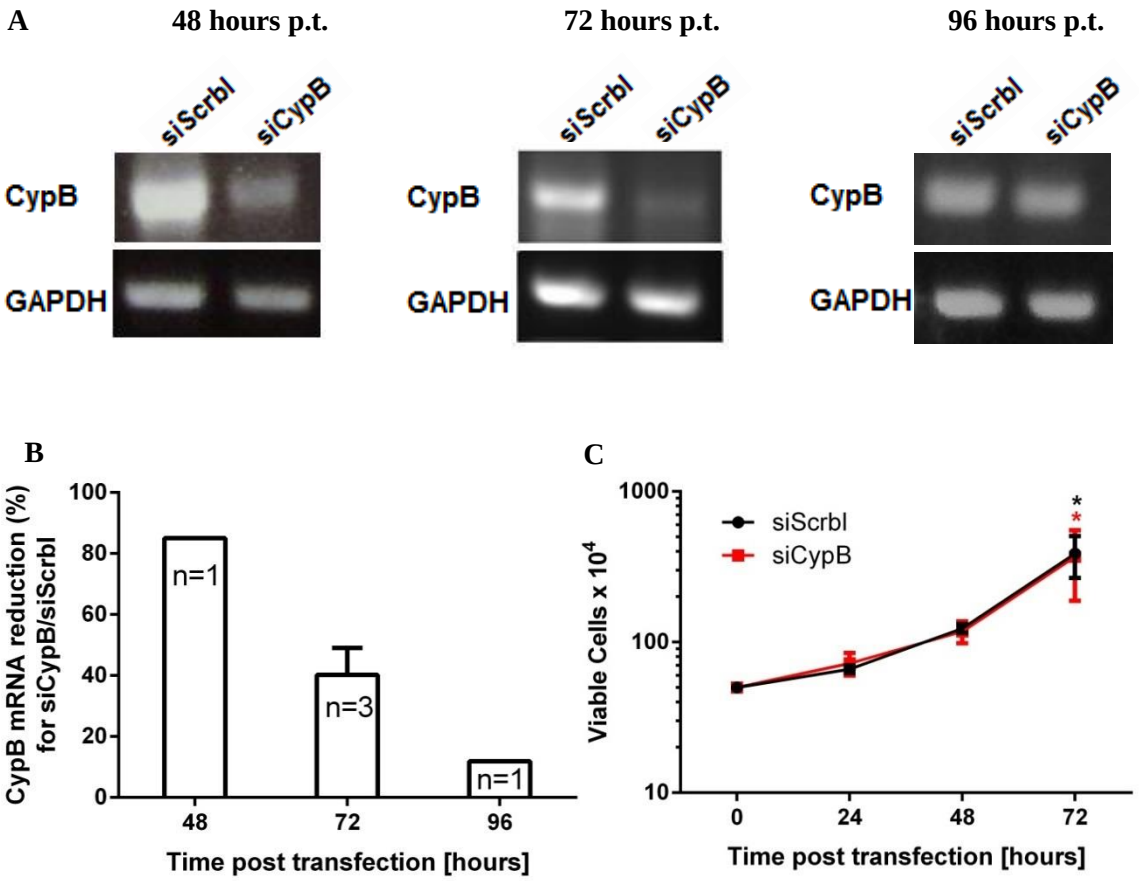


Figure 57: ***mRNA Expression of CypB in mCMP is Transiently Modulated by RNAi.*** mCMP were transfected with a siRNA pool for *mCypB* (siCypB) or a non-targeting control pool (siScrbl) with the Amaxa Nucleofector (as described in chapter 2.3.6.). mRNA was extracted from mNØ 48 -96 hours post transfection using the TRI method (as described in chapter 2.3.1.). Total cellular RNA was reverse transcribed into cDNA using random primers and reverse transcribed RT-PCR products (as described in chapter 2.) were visualised by ethidium bromide staining on a 1.2 % Agarose TAE gel (Panel **A**). *mCypB* levels were reduced by 85 % at 48 hours posttransfection (n=1), 52.5 % ± 13.6 SEM (n=3) at 72 hours p.t. and 12 % at 96 hours p.t. (n=1) compared to a non-targeting control and normalised to GAPDH (Panel **A**, **B**). Viable cell numbers for siScrbl and siCypB transfected cells were measured by haemocytometer counts. Viable cell numbers significantly increased for both siScrbl and siCypB transfected cells (n=3), whereas no differences in viability could be detected between the cultures (Panel **C**). The molecular weight standard HyperladderIV was used to approximate the band size of *mCypB* (343 bases) and *mGAPDH* (233 bases). Reductions in band intensity were quantified by densitometry with the ImageJ software. Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests and significant differences to the 0 hour control were indicated by asterisks (*). Results were considered to be statistically significant for $p<0.05$ (*).

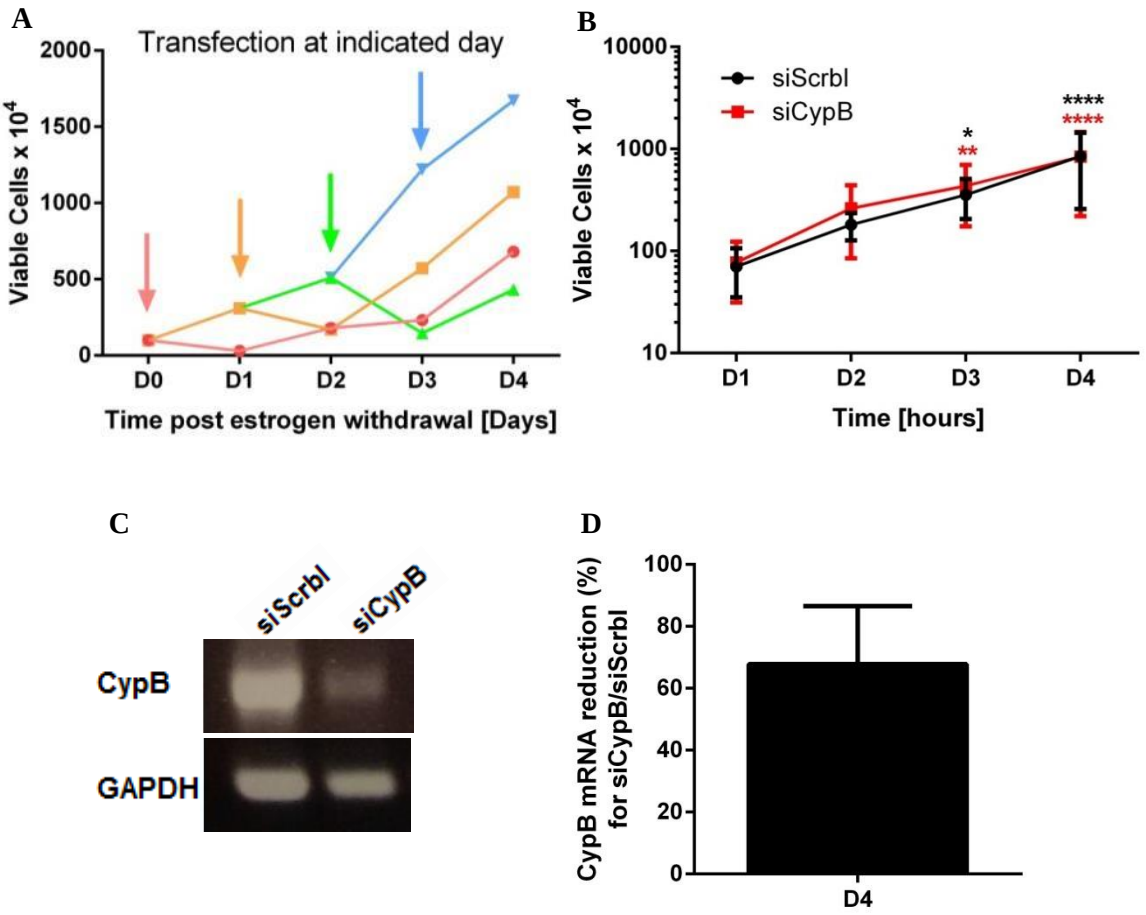


Figure 58: ***Transfection with siRNA for CypB in mCMP Modulates Gene Expression in mNØ.*** mCMP were incubated in differentiation medium for 4 days after estrogen withdrawal (as described in chapter 2.1.2.). mCMP were transfected on Day 0 (Panel **A**), Day 1 (Panel **A**, **B**), Day 2 (Panel **A**) or Day 3 (Panel **A**) with a siRNA pool for mCypB (Panel **B**) or the non-targeting control pool siScrbl (Panel **A**, **B**) using the Amaxa Nucleofector. mRNA was extracted from mNØ on D4 using the TRI and reverse transcribed into cDNA using random primers, where indicated. RT-PCR products (as described in chapter 2) were visualised by ethidium bromide staining on a 1.2 % Agarose TAE gel (Panel **C**). Transfection with siScrbl (Panel **A**) reduced viable cell numbers to a greater extent by transfection of D2, in contrast to D1 (n=1). Transfection of differentiation cultures on D1 of differentiation allowed the further expansion of the growth culture (Panel **B**), with no significant differences in viable cell numbers between siScrbl and siCypB transfected cells. On D4, *mCypB* levels were reduced by 67.8 % (± 18.8 SEM) in siCypB transfected cells in comparison to the non-targeting control (Panels **C**, **D**). Molecular weight standard HyperladderIV was used to approximate the band size of *mCypB* (343 bases) and *mGAPDH* (233 bases). Images were analysed with ImageJ. Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests. Unless otherwise indicated, data shown are representative for three independent experiments. Significant differences to D0 were indicated by asterisks (*). Results were considered to be statistically significant for $p<0.05$ (*), $p<0.01$ (**) and $p<0.0001$ (****).

5.3.2. Modulation of *Mcl-1* and *Casp-3* Levels in mNØ and Assessment of Functional Consequences.

Following the successful knockdown of *CypB*, it was aimed to modify the expression of key genes in neutrophil survival and apoptosis, and to study the functional consequences on cell death. In this context, caspase 3 (Casp-3) was chosen as a target, as it is a well-established component of spontaneous apoptosis (Pongracz *et al.*, 1999; Porter, Jänicke, 1999). In addition, the anti-apoptotic Bcl-2 family member Mcl-1 has been shown to be essential for neutrophil survival and therefore an ideal candidate for this experiment (Wardle *et al.*, 2011; Murphy, Caraher, 2015). Consequently, mCMP were transfected on D1 of differentiation with siRNA pools for the pro-apoptotic Casp-3 or Mcl-1. On D4, *mMcl-1* levels were reduced by 63.2 % (± 16.6 SEM) and *mCasp-3* levels by 57.5 % (± 11.7 SEM) in comparison to the non-targeting control (**Fig. 59A**, **B**). Viable cell numbers significantly increased in a time dependent manner in all transfection cultures during their differentiation (**Fig. 59C**). However, siMcl-1 transfected cultures had significantly lower viable cell numbers in comparison to the non-targeting control, indicating that *Mcl-1* knockdown cultures had a growth disadvantage. Transfection cultures showed a fully mature morphology on D4 (**Fig. 59D**), indicating that the reduction in mature mNØ numbers was not caused by a delay in differentiation. Therefore, it was hypothesised that changes in constitutive apoptosis in the *Mcl-1* knockdown culture decreased viable cell numbers during mNØ differentiation.

Consequently, fully mature mNØ from a 4 day differentiation culture were examined for baseline apoptosis. Transfected mNØ were cultured in basal medium for 6 or 30 hours. Viable cell numbers and apoptosis were examined by flow cytometry. At 0 hours, the mean percentage of $FL2^{+}/FL4^{-}$ cells in the *Mcl-1* knockdown culture was increased by 40.3 % ± 50.3 SEM in comparison to the non-targeting control (**Fig. 60A**), while $FL2^{+}/FL4^{-}$ cells in the *Casp-3* knockdown culture was decreased by 16.5 % ± 7.2 SEM, indicating that *Mcl-1* and *Casp-3* knockdown altered mNØ apoptosis. For all, siScrbl, siMcl-1 and siCasp-3 mNØ, the mean percentage of $FL2^{+}/FL4^{-}$ was significantly increased at 6 hour and 30 hours (**Fig. 60B**). At 30 hours, siCasp-3 transfected cells showed a significantly lower mean percentage of $FL2^{+}/FL4^{-}$ cells in comparison to siScrbl cultures. Viable cell numbers decreased over time for siScrbl transfected cultures (**Fig. 60C**). An increased number of viable cells were present in the *Casp-3* knockdown cultures compared to *Mcl-1* cultures, but this was not significant. Taken together, siMcl-1 and siCasp-3 mNØ displayed functional alterations in the regulation of their lifespan, and the increased apoptosis in siMcl-1 cultures might account for the decrease in viable cell numbers during differentiation.

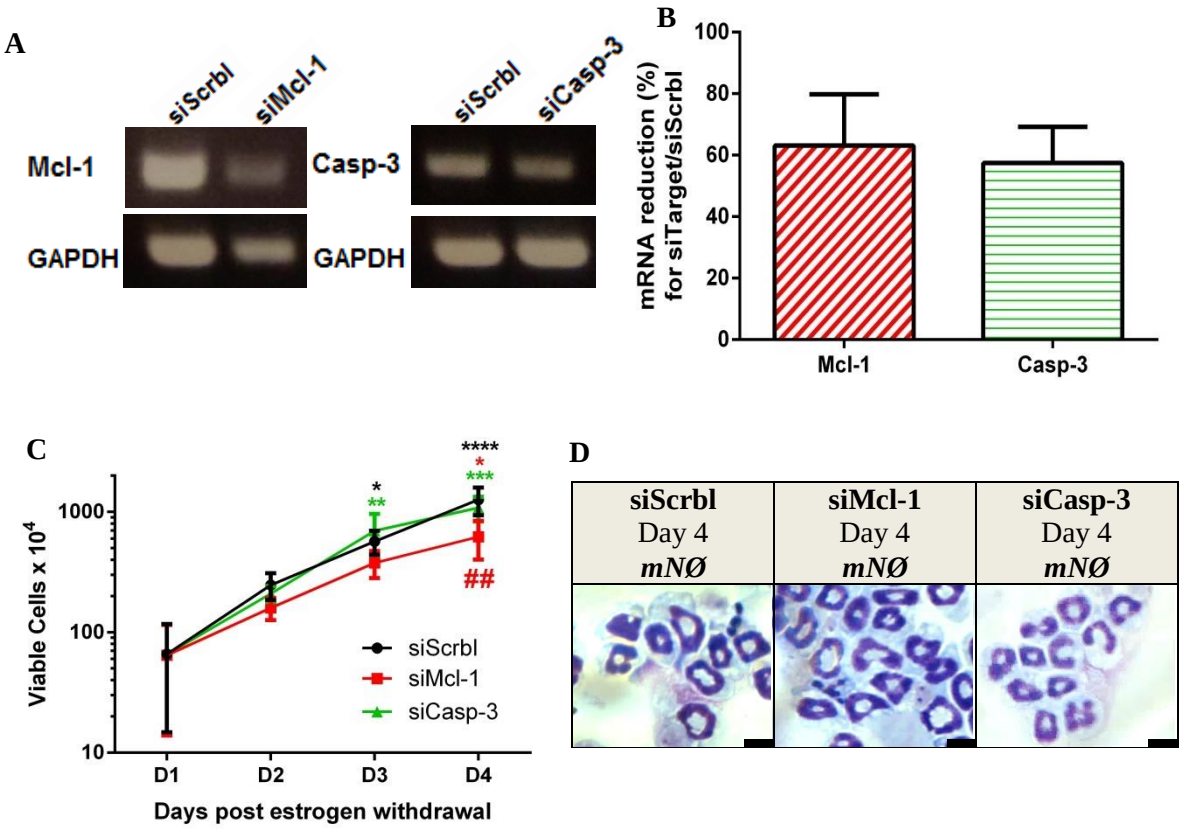


Figure 59: ***RNAi in mCMP Reduces Mcl-1 and Casp-3 mRNA Expression in Mature mNØ.*** mCMP were incubated in differentiation medium for 4 days after estrogen withdrawal (as described in chapter 2.3.6.). mCMP were transfected on Day 1 post estrogen withdrawal (Panel **A**, **B**) with a siRNA pool for mMcl-1, mCasp-3 or the non-targeting control siScrbl with the Amaxa Nucleofector. mRNA was extracted from mNØ on D4 using TRI reagent and reverse transcribed into cDNA with random primers. RT-PCR products (as described in chapter 2) were visualised by ethidium bromide staining on a 1.2 % Agarose TAE gel (Panel **A**). On D4, *mMcl-1* levels were reduced by 63.1 % (± 16.6 SEM) and *mCasp-3* levels by 57.5 % (± 11.7 SEM) in comparison to the non-targeting control (Panels **A**, **B**). Viable cell numbers of transfection cultures significantly increased in a time dependent manner during their differentiation (Panel **C**). siMcl-1 transfected cultures had significantly lower viable cell numbers in comparison to the non-targeting control. Transfection cultures showed a fully mature morphology on D4 (Panel **D**). Molecular weight standard HyperladderIV was used to approximate the band size of *mMcl-1* (265 bases), *mCasp-3* (379 bases) and *mGAPDH* (233 bases). Images were analysed with ImageJ. Statistical analysis was performed through Two Way RM ANOVA with Sidak's posttests. Data shown are representative for three independent experiments. Significant differences to D0 were indicated by asterisks (*), while changes to the siScrbl transfected culture were denoted by octothorpes (#). Results were considered to be statistically significant for $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****).

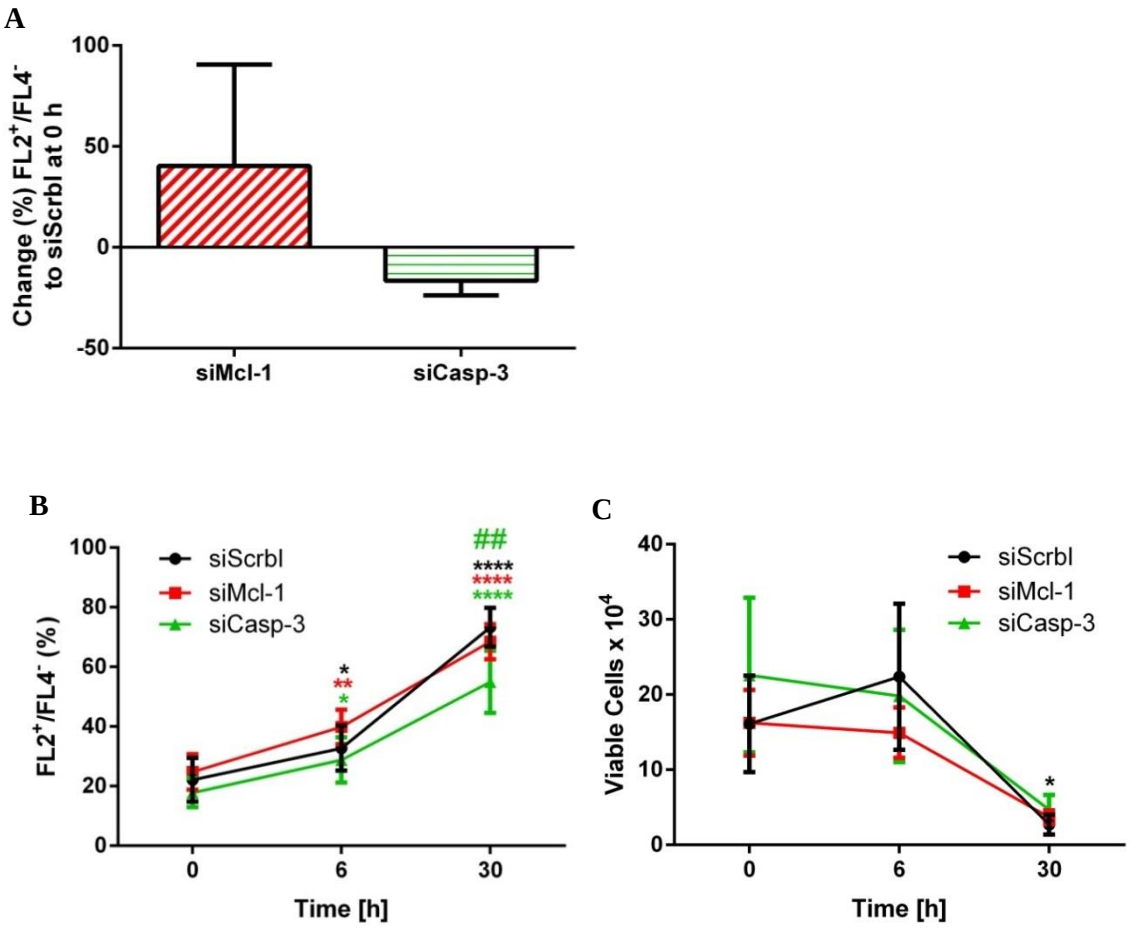


Figure 60: ***Modulation of Mcl-1 and Casp-3 mRNA Expression Induces Functional Changes in mNØ Apoptosis and Survival.*** mCMP were incubated in differentiation medium for 4 days after estrogen withdrawal (as described in chapter 2.3.6.). mCMP were transfected on Day 1 post estrogen withdrawal (Panel **A**, **B**) with a siRNA pool for mMcl-1, mCasp-3 or the non-targeting control siScrbl with the Amaxa Nucleofector. Modified mNØ from a 4-day differentiation culture were seeded at 3.3 x 10^6/ml apoptosis medium, as described in chapter 2.1.2. mNØ were cultured for 0 – 30 hours before apoptosis was quantified by FACS through PE-annexin V (FL2) and Topro-3 (FL4; Panel **B**) and total cell numbers determined through FACS analysis of CountBright beads (Panel **C**). At 0 hours, the mean percentage of $FL2^+/FL4^-$ cells in the Mcl-1 knockdown culture was increased by 40.3 % ± 50.3 SEM in comparison to the non-targeting control (Panel **A**), while $FL2^+/FL4^-$ cells in the Casp-3 knockdown culture was decreased by 16.5 % ± 7.2 SEM. Mean percentage of $FL2^+/FL4^-$ siScrbl, siMcl-1 and Casp-3 mNØ (Panel **B**) was significantly increased at 6 hour and 30 hours. At 30 hours, siCasp-3 transfected cells showed a significantly lower mean percentage of $FL2^+/FL4^-$ cells in comparison to siScrbl cultures. Viable cell numbers decreased over time for siScrbl transfected cultures (Panel **C**). Viable cell numbers of Casp-3 knockdown cultures were non-significantly increased. Data shown are mean ± SEM of 4 independent experiments. Statistical analysis was performed through Two Way ANOVA with Sidak's posttest. Significant differences to 0 hours were indicated by asterisks (*), while changes to the siScrbl transfected culture were denoted by octothorpes (#). Results were considered to be statistically significant for $p<0.05$ (*), $p<0.01$ (**/##) and $p<0.0001$ (****).

5.3.3. Modulation of *NR4A2* and *NR4A3* Levels in mNØ.

To examine the influence of nuclear receptors on mNØ lifespan, mCMP were transfected with a siRNA pool for *mNR4A2*, *mNR4A3* and a non-targeting control on D1 of differentiation. In mNØ, *mNR4A2* and *mNR4A3* levels were reduced at D4 by a mean of 91.2 % ± 3.4 SEM and 75.1 % ± 14.5 SEM in comparison to siScrbl mNØ respectively (**Fig. 61A**, **B**). Transfection with siNR4A2 in mCMP significantly reduced total viable cell numbers of mNØ at D4 (**Fig. 61C**), indicating a potential role of NR4A2 in mNØ development. Reasons for this include decreased differentiation, delayed proliferation or increased apoptosis, overall resulting in a growth disadvantage in contrast to siScrbl mNØ. Interestingly, an increased amount of $FL2^{+}/FL4^{-}$ mNØ was observed in *NR4A2* KD mNØ at D4 of differentiation (n=1; **Fig. 61D**).

5.4. Discussion

5.4.1. Choice of Apoptosis Assay.

The main aim of this chapter was to validate mNØ as a tool to investigate apoptosis and survival pathways and to be able to relate the findings to PMN survival pathways. For this end, the choice of an appropriate apoptosis assay is crucial to accurately distinguish distinct forms of cell death. Complicating their distinction is the fact that apoptotic cells are rapidly phagocytosed *in vivo*, while the lack of macrophages *in vitro* allows apoptosis to proceed to secondary necrosis (Haslett, 1999). Hence, in a neutrophil cell line with prolonged time in culture, this full cellular disintegration can lead to the culmination of extensive amounts of cellular debris.

Morphological assessment of apoptosis is often referred to as the most accurate measure of apoptosis and often described as the gold standard. This technique is based on microscopic observation of visible apoptotic hallmarks, such as chromatin condensation and cleavage, cell shrinkage and formation of membrane protrusions (Saraste, 1999). Murine neutrophils possess a nuclear shape distinct to that of human neutrophils, which can be observed by light microscopy (Thompson et al., 2014). Good practice for the assessment of apoptosis in murine neutrophils encompasses the use of flow cytometry to validate findings for morphological apoptosis. Assessing apoptosis solely based on their morphological features can be misleading, as mNØ displayed the first pre-apoptotic changes (i.e. localised spots of chromatin condensation) shortly after their differentiation (**Fig. 46A**), while they still possessed full membrane integrity, no PS exposure, or reductions in their forward and side scatter (FSC, SSC; **Fig. 48**).

However, the percentage of morphologically apoptotic mNØ was considerably lower (20 %, 36 hours; **Fig. 47A**) than mean mNØ apoptosis based on membrane integrity and PS exposure (40 %, 36 hours; **Fig. 48A**). Expression of PS on the outer membrane is an early apoptotic feature (Fadeel *et al.*, 1998; Fadok *et al.*, 2001; Hampton *et al.*, 2002), and thus may account for the higher levels of $FL2^{+}/FL4^{-}$ mNØ as compared to morphologically apoptotic cells. Moreover,

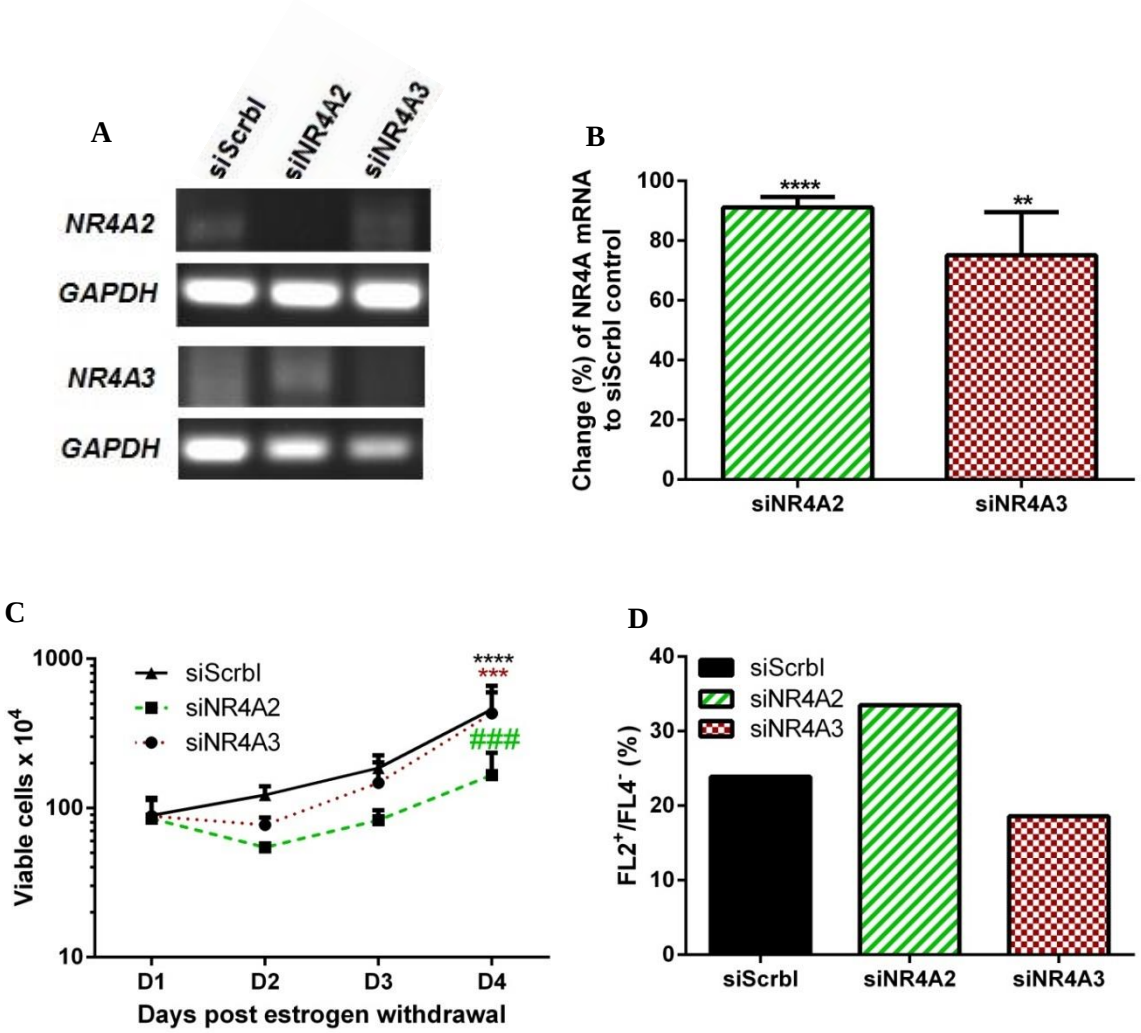


Figure 61: ***NR4A2, but not NR4A3 Knockdown in mCMP Reduces Cell Numbers During the Differentiation to mNØ.*** mCMP differentiation cultures were transfected one day post-estrogen withdrawal using the Amaxa Nucleofector. Cells were transfected with siRNA for *mNR4A2*, *mNR4A3* or a non-targeting control (*siScrbl*) on day 1, and subsequently incubated in differentiation medium for further 3 days with daily media replenishment. mRNA was extracted on day 4 using the TRI method (as described in chapter 2.3.1.). Total cellular RNA was reverse transcribed into cDNA using random primers. RT-PCR products (as described in chapter 2) were visualised by ethidium bromide staining of a 1.2 % Agarose TAE gel (Panel **A**). Total cell numbers were determined through haemocytometer counts (Panel **C**). *mNR4A2* and *mNR4A3* levels were reduced by 91.2 % ± 3.4 SEM and 75.1 % ± 14.5 SEM respectively (Panel **A**, **B**) at day 4 in mNØ. Representative blots are shown in Panel **A**. Transfection with siNR4A2 in mCMP significantly reduced total cell numbers of mNØ at D4 (Panel **C**). Molecular weight standard HyperladderIV was used to approximate the band size of *mNR4A2* (990 bases), *mNR4A3* (328 bases) and *mGAPDH* (233 bases). cDNA band intensity was quantified by densitometry using the ImageJ software. Data shown are mean ± SEM (Panels **B**-**D**), or representative images (Panel **A**) of 1(Panel **D**) or 3 (Panels **A**-**C**) independent experiments. Statistical analysis was performed through Two-Way ANOVA with Sidak's post-tests. Significant differences to D1 were indicated by asterisks (*), while changes to the siScrbl transfected culture were denoted by octothorpes (#). Results were considered to be statistically significant for $p<0.01$ (**), $p<0.001$ (***/###) and $p<0.0001$ (****).

neutrophils undergo secondary necrosis upon failure of efferocytosis *in vitro* (Haslett, 1999). This may lead to the underestimation of apoptosis in cultured cells, when relying on morphology alone (**Fig. 47**; **Fig. 48**). The gating used for this study relied on the use of negative controls for PS/Annexin V binding and membrane integrity (as described in chapter 2.2.2.). Interestingly, the negative control for membrane integrity was not only highly $FL4^+$, but also $FL2^+$, indicating that necrotic cells may expose PS residues and thus bind to Annexin V. This is supported by studies, where primary necrotic cells displayed PS/Annexin V binding and, albeit less efficiently than apoptotic cells, were phagocytosed by macrophages (Fadeel *et al.*, 1998; Fadok *et al.*, 2001; Hampton *et al.*, 2002; Broukaert *et al.*, 2004; Appelt *et al.*, 2005). Since PS is typically present on the internal plasma membrane leaflet, the increased membrane permeability may thus allow for the internalisation and binding of Annexin V to PS. In this study, dual positive cells with high levels of $FL4^+$ were excluded to allow the distinction between apoptotic and necrotic cells. A potential overlap of primary and secondary necrotic mNØ does not hinder the investigation of mNØ cell death in this study and is in fact comparable to the occurrence of secondary necrosis in human PMN.

It has been reported that membrane permeabilisation is not a unique feature of necrotic cells, but also occurs at late apoptotic/secondary necrotic stages (Dive *et al.*, 1992; Ni *et al.*, 1993; Ormerod *et al.*, 1993), and apoptotic cells allow increased leakage of viability dyes (Ormerod *et al.*, 1993; Schmid *et al.*, 1994; Idziorek *et al.*, 1995). This is consistent with the typically moderately increased FL4 fluorescence of $FL2^+$ cells in comparison to viable cells ($FL2^-/FL4^-$; **Fig. 48E**), suggesting that loss of membrane integrity encompasses later stages of apoptosis in mNØ. Hence, quantitatively more accurate means to determine actual amounts of neutrophils committed to an apoptotic pathway *in vitro* may incorporate the flow cytometric assessment of apoptotic features, where on average 30x more individual cells are routinely assessed per sample. It was therefore determined that for the purpose of this project, the quantification of murine apoptotic neutrophils can be more representatively undertaken through flow cytometric analysis.

Additionally, the quantification of cell loss in culture was used to provide another distinction between cell death mechanisms specifically modulating apoptosis or general cell survival. In this context, cell loss is an accumulative measurement, while apoptosis reflects more accurately apoptotic cells at a given time due to secondary necrotic loss of apoptotic cells. *In vitro* secondary necrotic cell loss furthermore results in variability if assessing cell loss based on total cell counts. Thus, cell loss can be more accurately reflected when based on the amount of viable cells remaining in culture (**Fig. 48C**). Viable cells can either be counted in a haemocytometer, or based on the relative abundance of flow cytometric beads in a sample. Hodge et al. (2004) previously demonstrated that neutrophil numbers determined by manual counting or flow cytometric assessment were comparable. In this context, the use of CountBright beads has the

convenience of linking the apoptotic cells in one sample directly to the cell counts. This measurement is independent from loss of culture volume due to evaporation. Therefore, viable cells were assessed by CountBright beads in this study, as this was perceived to be highly accurate and the most reproducible measure.

5.4.2. mNØ Apoptosis Displays Vital Similarities to PMN Apoptosis.

The similarity of mNØ to primary neutrophils was demonstrated by the occurrence of spontaneous apoptosis in a time-dependent manner (**Fig. 47**, **Fig. 48**). The apparent difference between morphological apoptosis and $FL2^{+}/FL4^{-}$ cells (**Fig. 47A, D**; **Fig. 48A, D**) may be explained by the longer preparation time and exposure to potentially cytotoxic reagents in the flow cytometry staining protocol. However, the previous assay found only a small difference between $FL2^{+}/FL4^{-}$ and all $FL2^{+}$ cells, indicating that necrotic cell death in itself is not a common occurrence. In fact, the lack of constitutive necrosis may also indicate the occurrence of secondary necrosis of apoptotic cells, and further limitations of the distinct assays were discussed in the previous subchapter. Briefly, a comparison between the apoptosis measurements for 36 hours showed over 40 % of cells were positive for PS exposure, while only 20 % of cells displayed apoptotic morphology at the same timepoint (**Fig. 47A, D**; **Fig. 48A, D**). The difference between the measurements is not unexpected, as PS exposure has been described as a hallmark of early apoptosis (Savill, 1997b), and therefore the measurement of PS exposure might be higher than morphological apoptosis at comparable timepoints. In a previous publication (Kirschnek *et al.*, 2011; Gautam *et al.*, 2013), considerably higher percentages of Annexin V/PI positive mNØ were observed at basal levels (20-40 %) and 24 hours (80-90 %), indicating that the occurrence of necrosis and PS exposure may vary considerably in different studies.

Moreover, the relative decrease in cell numbers from 0 hours to 12 hours was comparable for both assay types (**Fig. 47B**, **Fig. 48B**). Therefore, the most likely explanation is that basal levels of cell loss occurred due to the washing steps during preparation and their analysis in the flow cytometer, which is a common limitation of flow cytometric analysis (Dux *et al.*, 1994).

mNØ apoptosis was modulated by addition of growth factors (**Fig. 49**), corresponding to the findings in primary human neutrophils (van Raam *et al.*, 2008). At 24 hours, a third of mNØ were apoptotic in growth-factor deprived media, whereas a quarter was apoptotic in presence of SCF and G-CSF (**Fig. 49A**). This correlates well with the apoptosis rates in bone-marrow derived murine neutrophils with 70 % survival in growth factor deprived media, and 80 % survival in supplemented medium at the same timepoint (Villunger *et al.*, 2003) and mNØ apoptosis of 53.2 % in absence of SCF and 16.9 % in presence of the growth factor at 24 hours (Koedel *et al.*, 2009). In human PMN, 45 % (Percoll-purified PMN) to 70 % (Optiprep- highly purified PMN) apoptosis would be expected at a comparable (20 h) time point (Wardle *et al.*,

2011). Apart from species-dependent variations in neutrophil lifespan, these differences in constitutive apoptosis rates might also depend on the initial neutrophil maturity and cell age at start of the experiments. Thereby, bone-marrow derived murine neutrophils resemble mNØ closely. This is interesting, as it has not only been shown that the occurrence of band neutrophils is more common in murine blood, but also that Percoll gradients can be utilised to separate different types of neutrophil precursors according to their increasing densities in maturation (Cowland, Borregaard, 1999). Potentially this might also account for differences between Percoll and Optiprep-purified neutrophils.

However, it cannot be disregarded that the occurrence of uncommitted mCMP by the end of the differentiation process might account for the presence of functional macrophages and thus reduced numbers of apoptotic neutrophils in samples; the importance of this factor was previously demonstrated (Sabroe *et al.*, 2001; Sabroe *et al.*, 2004). Based on the low presence of mCMP, macrophages, and band neutrophils in light microscopic examination of differentiation cultures (**Fig. 46**), the effect of this phenomenon is likely to be rather limited. Additionally, the further purification of differentiation cultures by a modified Percoll column (data not shown), which would remove contaminating mononuclear cells, did not result in substantial increases in cell purity and constitutive apoptosis.

Kirschnek *et al.* (2011) observed that ER-Hoxb8 mNØ show typical high rates of constitutive apoptosis (80% at 24 hours) in culture. This is surprising, as murine neutrophils typically display lower rates of apoptosis than human neutrophils (Villunger *et al.*, 2003). In our study, we have found rates of 30% apoptosis at 24 hours (**Fig. 49**), comparable to the rates previously observed for murine bone-marrow neutrophils (Villunger *et al.*, 2003), and consistent with the results of Kirschnek *et al.* (2011) for bone marrow derived mNØ. In summary, differences in apoptosis rates found in this study were only subtle in comparison to primary murine neutrophils, while differences to human PMN were more pronounced. Culturing conditions of differentiated neutrophils were aimed at modelling physiological conditions, where a majority of lobulated neutrophils are released into the blood stream following their maturation in the bone marrow (Metcalf, 2008). Taken together, it was concluded that SCF and G-CSF suppress mNØ apoptosis and prolong their lifespan to a limited extend at late timepoints. However, mNØ retained their ability for constitutive apoptosis even in presence of growth factors. Moreover, the residual amount of growth factors in the culture after consecutive washes is likely to be extremely low. Hence, the influence of SCF and G-CSF in this setting is thought to be negligibly low.

Apoptotic cells *in vitro* undergo secondary necrosis, which is thought to be due to the lack of macrophage phagocytosis (Haslett, 1999). Here, the observed cell loss in **Fig. 47** correlated well with the apoptosis levels and it was thus concluded that apoptosis constitutes the major form of cell death occurring in this model system (**Fig. 47**). Both, intrinsic and extrinsic apoptosis

essentially rely on the action of initiator and executioner caspases. Hence, the pan-caspase inhibitor Q-VD-OPh (QVD) was utilised to show the relevance of caspases in mNØ apoptosis. Consistent with the reported effect of Q-VD-OPh on primary human neutrophils (Wardle *et al.*, 2011) and a previous study in mNØ (Kirschnek *et al.*, 2011), apoptosis was significantly delayed by QVD, demonstrating the caspase dependence of mNØ apoptosis (**Fig. 50**). However, QVD more effectively induced apoptosis in a comparable study with human PMN (Wardle *et al.*, 2011). This may indicate that affinity of QVD for murine caspase domains may be reduced. Interestingly, mice lack the initiator caspase 10 (Lamkanfi *et al.*, 2002), which may account for the difference in efficacy. However, in human cells, QVD was equally effective at targeting the different caspase domains (Caserta *et al.*, 2003). On the other hand, QVD might also cause nonspecific effects, as demonstrated by its (limited) cellular toxicity in high concentrations with human neutrophils (Caserta *et al.*, 2003). This would not be surprising, as various other caspase-inhibitors are poorly selective and show wide-ranging toxic effects (McStay *et al.*, 2008). Interestingly, there are a large number of caspases, with partially redundant functions involved in human apoptosis pathways (McStay *et al.*, 2008). In murine neutrophils, homologues for most human caspases exist; however, existing differences between two species (reviewed by: Mestas *et al.*, 2004; Degterev *et al.*, 2003) may account for discrepancies in the responses to QVD. Due to the high divergence in human caspases, the question of partial functional abundance might become relevant, as apoptosis can be engaged in a similar manner in both systems.

Treatment of mNØ with the broad-spectrum protein kinase inhibitor staurosporine resulted in a significant increase in late apoptosis (**Fig. 51**). The low efficiency of STS is not surprising, as the molecular action of staurosporine in apoptosis is not well understood, but may only partially be derived by the activation of caspases (Belmokhtar *et al.*, 2001), furthermore supporting the caspase-dependence of mNØ apoptosis. Besides its action on caspases, staurosporine competes for ATP-binding and thereby acts as a pan-kinase inhibitor, which might indicate that staurosporine shows a lower efficacy in mNØ, because important pro-survival kinases, such as PKA, are not constitutively active. Taken together, STS increased apoptosis, indicating that either constitutive kinase activity or caspase dependent apoptosis are involved in the regulation of mNØ lifespan.

5.4.3. The Relevance of PKA Signalling in mNØ Survival.

The relevance of PKA survival pathways in mNØ was evaluated. Thereby, the cAMP agonist dbcAMP and the PKA agonist pair N6-MB-cAMP and 8-AHA-cAMP were shown to reduce apoptosis (**Fig. 52**, **Fig. 53**). Reduction in apoptosis for dbcAMP in mNØ at 30 hours was comparable to percentages observed at 20 hours for human PMN (**Fig. 36**; Martin *et al.*, 2001). This might indicate that human PMN are more prone to undergo apoptosis, potentially due to

their mixed age profile at the start of the experimental setup *in vitro*. Use of N6/8-AHA (**Fig. 53**) more efficiently reduced apoptosis than dbcAMP (**Fig. 52**), consistent with the earlier finding in PMN (**Fig. 28-29**; discussed in chapter 4.3.). Briefly, an explanation for the lower efficacy of dbcAMP may be due to its poor cell permeability, the potential side-effects of second signalling chains induced by butyrate (Yusta *et al.*, 1988). Interestingly, dbcAMP shows a low activity towards PKA in its inactive form (Yusta *et al.*, 1988), which may reduce its activity in mNØ survival pathways. Interestingly, viable cell numbers of dbcAMP treated cells were increased at 6 hours of treatment (**Fig. 52B**). As mNØ are postmitotic and the number of progenitors in the cell preparations was negligibly low, a possible explanation may be that dbcAMP could exert an effect on cell numbers at the 0 hours timepoint already during the time required to prepare cells for their flow cytometric assessment.

The PKA antagonist Rp-8-Br-cAMPS only increased basal mNØ apoptosis by a small extent and had no effect on viable cell numbers (**Fig. 54**), indicating that only a low level of constitutive PKA signalling might be active in mNØ. A technical reason for the low efficiency of Rp-8-Br-cAMPS in the induction of mNØ apoptosis might involve a potentially decreased affinity of Rp-8-Br-cAMPS to murine PKA isoforms. However, as mNØ are not directly derived from an *in vivo* system, and thereby potentially show but a low level of constitutive PKA-dependent survival due to a lack of previous cell activation. In **fig. 52**, PKA survival was readily engaged by dbcAMP, and cell numbers were increased at 6 hours (**Fig. 53B**), and late mNØ apoptosis was increased by Rp-8-Br-cAMPS treatment (**Fig. 54A**). In **fig. 55B**, dbcAMP did not induce significant amounts of mNØ survival, which consequently could not be blocked by PKA antagonism. Despite the inefficacy of inducing survival, dbcAMP had a low effect on reducing apoptosis (**Fig. 55A**), which was not prevented by Rp-8-Br-cAMPS treatment. This apparent inconsistency with previous data on PKA inhibition in mNØ may very well indicate that other cAMP-responsive pathways, such as Epac-signalling, have a greater influence on survival signalling in mNØ than in human PMN. Moreover, the rather low constitutive activity of PKA signalling (**Fig. 54**) may also differ between distinct batches of cells, which consistently also possess varying levels of constitutive apoptosis. Nevertheless, a near significant p-value of $p=0.0569$ was achieved by t-test, when comparing the media control to Rp-8-Br-cAMPS treatment samples. A comparison of Rp-8-Br-cAMPS treatment of dbcAMP treated samples only achieved a p-value of $p=0.1596$, which may further support the involvement of further cAMP-dependent signalling pathways that may alter apoptosis levels. The decreased efficiency of LPS and PGE2 in rescuing mNØ from apoptosis (**Fig. 56**) may be due to a decreased affinity of the agonists towards the murine receptor isoforms. Moreover, it is possible that the expression of the relevant pro-survival receptors in mNØ (i.e. EP receptors, TLR4) is diminished, and a minimum threshold level of receptor binding needs to be achieved before PKA activation is achieved. In support, B cells and macrophages from BALB/c mice exhibit

reduced functional responses to LPS stimulation caused by their attenuated TLR4 receptor expression (Tsukamoto *et al.*, 2013; Oliveira *et al.*, 2014a), caused by a single nucleotide polymorphism in the receptor gene. Additionally, the responsiveness of mice to LPS is greatly attenuated in comparison to humans (Warren *et al.*, 2010). In Balb/c mice, no constitutive expression of EP receptors EP1, EP2, EP3 and EP4 was detected (Akaogi *et al.*, 2004), while their expression could be induced. This may help to explain the reduced efficacy of PKA survival in mNØ.

As PKA survival can be engaged in mNØ (**Fig. 52-53**), it was concluded that the reduced efficacy of PKA antagonism in mNØ may be caused by 1) the low constitutive activity of PKA signalling, 2) the specific involvement of a PKA subtype, or 3) the unknown affinity of the antagonist towards PKA homologs in mice. However, this does not necessarily reflect upon the physiological relevance of the model in the research of PKA survival. Taken together, only limited evidence for the relevance of PKA-mediated survival in mNØ was determined, while this may be comparable to the signalling events in Balb/c mice and may be overcome by the use of a different background strain used in the production of mCMP. A failure to achieve significant changes in constitutive apoptosis through PKA antagonist treatment further indicate that only a negligibly low constitutive PKA activity might be present in mNØ.

5.4.4. Optimisation of Gene Expression Knockdown in mNØ.

Artificially synthesised siRNAs, targeting specific gene products can be used to specifically modulate gene expression of desired mRNAs and knock down gene expression in dividing cells (Tuschl *et al.*, 2002). These siRNAs are 19 base pairs double-stranded RNA molecules with an overhang of two bases. Hydrophobic bilayer membranes contain many hydrophobic pore openings, which can be converted into hydrophilic pores through an electric pulse, leading to rearrangements of the lipids in the pore composition, so that highly charged RNA is enabled to pass through (Tarek, 2005). According to the manufacturer, electroporation is ~10x more effective than chemical transformation, it can however also result in increased cell death. As mNØ are terminally differentiated and quiescent cells (Bainton *et al.*, 1971; Klausen *et al.*, 2004; Theilgaard-Monch *et al.*, 2005), the efficacy of RNAi needs to be empirically determined and optimised to achieve gene product knockdown in the course of the differentiation of mCMP to mNØ.

CypB is a non-essential housekeeping gene that is not involved in the maintenance of cell survival (Pachot *et al.*, 2004). Transfection of mCMP with a validated siRNA *CypB* control pool most efficiently reduced gene knockdown at 2 days post-transfection (**Fig. 57**), where siRNA transfected mCMP retain their viability and potential for growth. However, gene knockdown before the start of the differentiation of mCMP to mNØ (here: D0), resulted in a low number of viable neutrophils at day 2 post-estrogen withdrawal (**Fig. 58A**). In mCMP, a reasonable

reduction of *mCypB* levels (40 %) was observed 3 days post-transfection (**Fig. 57**). Therefore, mCMP were transfected on day 1 of the differentiation protocol, overall yielding a greater level of mNØ numbers on day 4 post-estrogen withdrawal (**Fig. 58**). Viable cell numbers were not significantly different from a control transfection with non-targeting siRNA (**Fig. 58B**), and *mCypB* levels were reduced by a considerable amount (67.8 % ± 18.8 SEM; **Fig. 58 C, D**). This adds to a study, where CypB was proposed as a general housekeeping gene in peripheral blood cells (Pachot *et al.*, 2004).

5.4.5. Modulation of the Expression of *Mcl-1* and *Casp3* mRNA.

To determine the applicability of mNØ as a model system for the modulation of survival and apoptosis pathway components, the pro-survival mediator Mcl-1 (Wardle *et al.*, 2011) and the pro-apoptotic caspase 3 (Pongracz *et al.*, 1999) were chosen as targets for gene expression knockdown in mNØ. siRNA transfection reduced *mMcl-1* mRNA by 63.2 % (± 16.6 SEM) and *mCasp-3* levels by 57.5 % (± 11.7 SEM), respectively (**Fig. 59**), with no apparent changes in the morphological phenotype of mature mNØ. In fact, gene knockdown increased constitutive apoptosis by 40.3 % (± 50.3 SEM) in *mMcl-1* KD mNØ and reduced basal apoptosis by 16.5 % (± 7.2 SEM) in mCasp-3 KD mNØ at 0 hours (**Fig. 60A**), consistent with the expected function of the molecules in the regulation of mNØ lifespan. Interestingly, in the course of the next 6 – 30 hours, apoptosis in *mMcl-1* KD mNØ was not significantly different to the non-targeting control. This indicates that Mcl-1 is not modulating mNØ survival following the activation of constitutive apoptosis pathways. This is in line with the observations of Wardle *et al.*, (2011), who showed that *Mcl-1* levels in human PMN decline in a temporal manner. Moreover, Mcl-1 can be cleaved following caspase activation, and caspase-inhibitable decreases in Mcl-1 expression were observed in late stages of apoptosis (Wardle *et al.*, 2011).

Consistently, at 30 hours, the mean levels of apoptosis in mCasp-3 KD mNØ were significantly decreased in comparison to the non-targeting control by close to 20 % (**Fig. 60B**). This suggests a role for caspase 3 in mNØ apoptosis, but a lower influence of the molecule in basal conditions. This is consistent with the reported involvement of Casp-3 in Noxa-dependent apoptosis in mNØ (Kirschnek *et al.*, 2011). Apoptosis was not completely prevented in Casp-3 KD mNØ, indicating that Casp-3 may be essential, but not sufficient to induce apoptosis. Consistently, the non-redundant roles of the effector caspases in the induction of apoptosis were previously demonstrated (Slee *et al.*, 2001). Interestingly, Casp-3 knockdown could not prevent the occurrence of apoptosis, but resulted in defects of nuclear fragmentation (Degterev *et al.*, 2003), consistent with the reported requirement for Casp-3 in apoptosis (Woo *et al.*, 1998).

5.4.6. Role of *NR4A2* mRNA during mNØ Differentiation.

In chapter 4.2.4, a role for the nuclear receptors NR4A2 and NR4A3 in PKA-dependent survival of PMN was observed. Therefore, the effect of *NR4A2* and *NR4A3* mRNA reduction by RNAi was investigated here. An effective knockdown of *NR4A2* and *NR4A3* levels by 91.2 % (± 3.4 SEM) and 75.1 % (± 14.5 SEM), respectively, was achieved at day 4 post estrogen withdrawal (**Fig. 61A, B**). The *NR4A2* KD mNØ culture showed significantly decreased cell numbers at the end of the differentiation process (D4; **Fig. 61C**), indicating that NR4A2 has important roles during mNØ differentiation. Consistently, specific roles for NR4A2 in cell development and differentiation have previously been reported (Yao *et al.*, 2012; Hawk, Abel, 2011; Sekiya *et al.*, 2013). Thus, NR4A2 may also be employed as an anti-neutrophilic strategy in COPD by reducing the numbers of pre-mature neutrophils in the blood during the immune response (Gossett and Macwilliams, 1982, Yokose *et al*, 1998; Seebach *et al.*, 1997).

Interestingly, apoptosis of *NR4A2* KD mNØ, but not *NR4A3* KD mNØ cultures at Day 4 of differentiation was ~20% higher than in siScrbl-transfected mNØ (n=1; **Fig. 61D**). This indicates that the decreased cell numbers during *NR4A2* KD mNØ differentiation may also be grounded in the increased rate of cellular apoptosis, as supported by a recent study on the role of NR4A2 in survival (Crean *et al.*, 2015).

5.5. Summary

In conclusion, the use of murine Hoxb8 conditionally immortalised progenitors and derived mNØ is a physiologically relevant model for research into neutrophil survival pathways and offers the potential to genetically manipulate and alter gene expression levels by siRNA interference in mCMP prior to their differentiation into mNØ. Murine models were used for several decades in granulocyte research, and a substantial amount of knowledge has been accumulated for the peculiarities of murine neutrophils and their conformities with human neutrophils. Furthermore, the model provides the unique opportunity to modulate gene expression levels by siRNA interference in neutrophils, which opens near unrestricted possibilities to investigate pathways underpinning neutrophil apoptosis and survival.

Despite the lack of PKA–mediated survival in mNØ, these cells showed responses similar to those observed in peripheral blood neutrophils, while being susceptible to manipulation of gene expression levels by non-viral RNAi techniques. While a proof-of-principle for the use of this technique has been provided here, the optimisation of the nucleofection strategy might enhance the gene expression level changes in differentiated neutrophils.

Moreover, a role for the nuclear receptor NR4A2 in mNØ development and potentially mNØ survival was uncovered. The use of a different mouse strain in the expression of ER-Hoxb8 in mCMP may thus be useful to further explore the role of NR4A2 in PKA-mediated survival.

CHAPTER 6. DISCUSSION

6.1. Major Findings of this Thesis

This thesis describes detailed examination of the receptors and molecular mechanisms of PGE2 survival in PMN in the context of COPD. The pro-survival effect of the prostaglandin PGE2 on PMN was shown to be exerted by the EP2 receptor subtype and through the activation of PKA, with only a minor for the EP4 receptor subtype on PMN survival. Despite a high affinity of PGE2 towards all prostaglandin receptors, PGE2 concentrations in the micromolar range were required to induce PMN survival. Possible explanations included the affinity of PGE2 on FP receptors, masking the pro-survival effect of PGE2, or that the amount of unbound PGE2 might be reduced by the presence of serum in the cell culture medium. Moreover, variations in the efficacy of PGE2-induced PMN survival may be introduced by the constitutive activity of PKA in PMN, which was in turn influenced by both, the presence of macrophages, as well as an autocrine survival signal.

Here, a novel mechanism for the connection of LPS and PGE2 signalling in PMN survival was uncovered. It was demonstrated for the first time that LPS modulates the expression of the AC-coupled prostaglandin receptors EP2 and EP4 in PMN (**Fig. 62**), which may sensitize PMN to the PGE2-mediated survival. Consistently, LPS induced the PKA-inhibitable expression of the PKA downstream targets *NR4A2* and *NR4A3*, correlating with LPS-induced survival.

However, in co-treatment experiments, PGE2 prevented LPS-induced prostaglandin receptor upregulation, suggesting a negative feedback loop in the regulation of PMN survival. The pro-survival influence of monocytes on PMN survival (Sabroe *et al.*, 2002a) and the greater influence of LPS on PMN survival in this context were substantiated. Thus, a crucial step in the sustenance of the inflammatory process and increased PMN survival may be through monocyte-derived PGE2 secretion, induced by LPS.

Prostaglandin receptor expression of EP2 and EP4 was comparable in PMN from healthy control and COPD patients. This furthermore substantiated current models, where neutrophils are considered the inflammatory mediators, but not initiator cells in COPD (Sabroe *et al.*, 2002b). The understanding of the causes of PGE2 secretion by other immune cells implicated in the pathology of COPD may thus be beneficial towards the understanding of the complex mechanisms of the inflammatory network in COPD.

Moreover, it was determined that the nuclear receptors NR4A2 and NR4A3 are downstream effectors of PKA signalling, and are induced by the EP2/PGE2 signalling axis, thus potentially coupling to PMN survival. In a murine neutrophil model, gene expression knockdown uncovered a functional role of NR4A2 in neutrophil development, potentially through a pro-survival role in neutrophils.

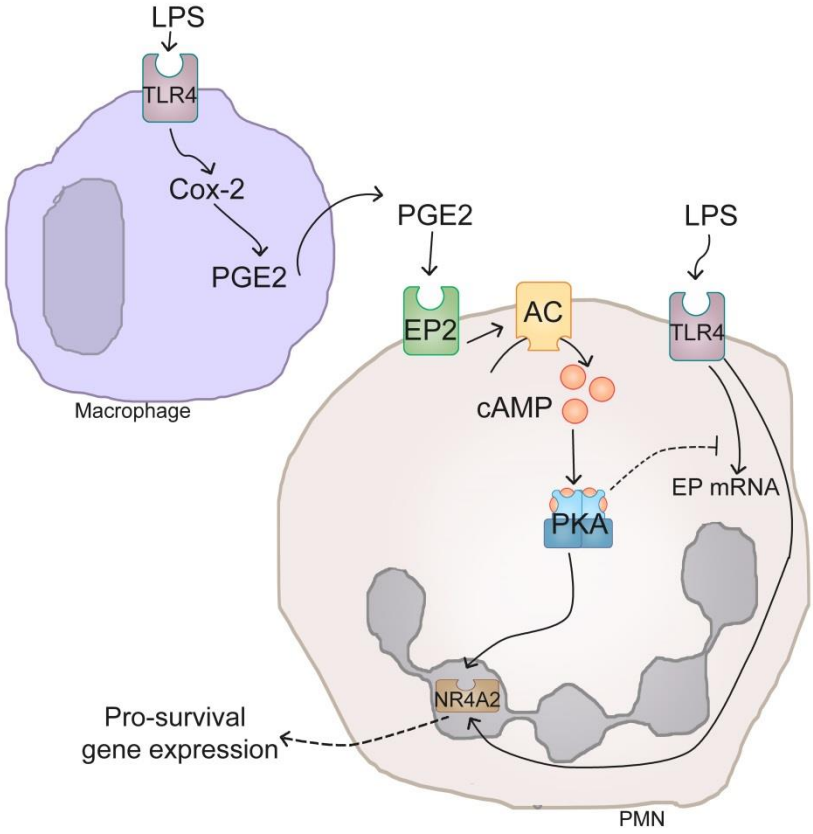


Figure 62: ***Proposed Interactions of LPS and PGE2 Signalling in the Induction of Neutrophil Survival in Inflammation in COPD.*** LPS prolongs PMN survival through several mechanisms: indirectly, through the induction of Cox-2 expression in macrophages, leading to the release of PGE2 into the inflammatory environment. Secondly, LPS increases the expression of EP receptor mRNA, which enhances the late response of PMN to PGE2, thus prolonging their survival. Thirdly, LPS-induced increases in NR4A2 expression were inhibited by PKA, which may present a switch from early to late survival pathways and/or to the resolution of inflammation. PGE2 in itself acts through PKA to induce NR4A2 mRNA expression, overall prolonging neutrophil survival, potentially through the induction of pro-survival gene expression. Abbreviations: LPS – lipopolysaccharide, TLR4 – toll-like receptor 4, Cox-2 – cyclooxygenase 2, PGE2 – prostaglandin E2, EP2 – e-prostanoid receptor 2, AC – adenylyl cyclase, cAMP – cyclic AMP, PKA – protein kinase A, PMN – polymorphonuclear cell.

6.2. Implications for the Regulation of PMN Survival Through PKA in Inflammation.

Current therapeutic strategies neglect and underestimate the harmful effect of neutrophils in inflammatory conditions. Neutrophils are considered to exert a protective role in inflammation due to anti-inflammatory cytokine expression (Tecchio *et al.*, 2014), whereas mounting scientific evidence of a pathogenic role for neutrophils in chronic inflammation likewise exists (Peleman *et al.*, 1999; Yoshioka *et al.*, 1995; Nadel, 1991; Qiu *et al.*, 2003; O'Donnell *et al.*, 2004; Baraldo *et al.*, 2004), and neutrophils are also known to express a range of pro-inflammatory cytokines (Tecchio *et al.*, 2014). In this context, neutrophilia is well known to contribute to the deterioration observed in acute exacerbations in chronic inflammatory conditions, such as COPD (Qiu *et al.*, 2003). Due to the fact that neutrophils are the main leukocyte associated with COPD, the regulation of neutrophil survival may be crucial towards the clinical outcome of COPD patients. Thus, the increased neutrophil presence may be a beneficial host-response at the early stages of infection and inflammation, but a failure in neutrophil clearance at later stages of the inflammatory response may contribute to the persistence of chronic inflammation.

Here, it was proposed for the first time that LPS may indirectly enhance PMN survival through the increased mRNA expression coding for EP2 and EP4 receptors, while the direct functional relevance through increased protein expression could not be demonstrated at the timepoints in question. Whereas basal expression of EP4 was greater in the neutrophil, PGE2 survival was mainly transduced by the action of EP2. Consistently, it was previously described in macrophages that a functional switch from EP4 to EP2 takes place in macrophage activation by LPS (Ikegami *et al.*, 2001). Simultaneously, increasing levels of LPS incapacitated constitutive PKA signalling, as PMN lost responsiveness to PKA inhibition. This may indicate that in the primary stages of acute inflammation, PMN become functionally prepared for the later stages of the inflammatory response, and their late survival may be enhanced through a PKA-independent mechanism. Moreover, macrophages are able to produce large quantities of PGE2 (Arakawa *et al.*, 1996). Interestingly, LPS previously induced the secretion of PGE2 in a Cox-2 dependent manner in monocytes at near-pharmacological levels (Amann *et al.*, 2001; Ikegami *et al.*, 2001; Hofer *et al.*, 2004); and the presence of PBMC also induced PMN survival (Sabroe *et al.*, 2002a). Increased levels of PGE2 may then potently enhance PMN survival through the LPS-induced EP2 and EP4 receptor protein expression, making them more responsive to the conditions present in inflammatory lesions. The subsequent recruitment of macrophages into the tissue and LPS-induced PGE2 secretion may further enhance PMN survival. Similarly, PGE2 regulates EP receptor expression by a potential negative feedback loop, ultimately predisposing the cells for increased apoptosis. In COPD PMN, LPS was more effective at the induction of

EP2 receptor expression, and the following hyperresponsiveness may enable neutrophils to linger at inflammatory sites.

To control the effect of PGE2, Cox-2 inhibitors were proposed as a pharmaceutical strategy in COPD (as discussed in chapter 1.2.2.4.). Despite showing potent anti-inflammatory potential, their use was linked to an increased risk of myocardial side-effects. This may argue for both a damaging, but also beneficial effect of PGE2 in inflammation. Consistently, the non-specific anti-inflammatory drug (NSAIDS) Ibuprofen, also acts as a Cox-2 inhibitor (Rose *et al.*, 2014). The results from this study may indicate that the effect of Cox-2 inhibition may partly be derived from the inhibition of PGE2-induced PMN survival. Interestingly, LPS and cigarette smoke highly increased cell numbers of neutrophils in EP4 knockout mice (Birrell *et al.*, 2015). This suggests that in addition to the stimulatory effect of PGE2 on PMN survival, it exerts a negative feedback effect, which serves to control unnecessary increases in PMN in neutrophilia. Thus, a therapeutic strategy may aim at the discovery of a survival-specific target of PGE2/PKA signalling in neutrophils.

In summary, neutrophil survival can be engaged by various stimuli, present in the context of inflammation, such as LPS and PGE2. Both stimuli can engage PMN survival through divergent pathways, with a common denominator being PKA signalling. This study showed a novel link between LPS and PGE2-induced PMN survival, which is an important step towards the understanding of the role of PKA-mediated PMN survival and neutrophilia in inflammatory diseases. A potential therapeutic strategy involving the targeting of the nuclear receptors NR4A2 and NR4A3 for the resolution of persistent neutrophilia in COPD will be discussed in the following chapter.

6.3. Therapeutic Strategies by Targeting Nuclear Receptors NR4A2 and NR4A3.

Targeting neutrophil apoptosis has often been proposed as a strategy to enable the resolution of inflammation (Heasman *et al.*, 2003; Kennedy, DeLeo, 2009; Sousa *et al.*, 2010). The value of neutrophils during the acute inflammatory response in the lung (i.e. anti-inflammatory cytokine expression, host defence; Tecchio *et al.*, 2014; Parker *et al.*, 2005) may be outweighed by the pathogenic/deleterious consequences of their persistence during the chronic inflammatory response (i.e. cytokine expression; tissue damage; elastase secretion; Tecchio *et al.*, 2014; O'Donnell *et al.*, 2004; Yoshioka *et al.*, 1995; Nadel, 1991) and a delay in their apoptosis, which increased the severity of COPD (Zhang *et al.*, 2012). Current therapeutic strategies are inadequate, as they may alleviate some of the symptoms, such as bronchoconstriction, but they cannot effectively reverse the disease progression. One particularly neglected characteristic of COPD is the excessive presence of neutrophils in the lung, although there is compelling evidence for their detrimental effects (as elaborated in section 1.1.).

A commonly employed non-invasive treatment options in COPD are bronchodilators to decrease the airflow resistance in the airways and steroids to reduce the swelling in the airways. β2-adrenoreceptor agonists are bronchodilators that moderately relieved bronchoconstriction, but did not significantly improve other characteristics of the disease (Appleton *et al.*, 2000). β2-adrenoreceptor agonists increase intracellular cAMP levels and thus activate PKA in SMC, resulting in airway relaxation. However, it was also shown that treatment with β2-adrenoreceptor agonists inhibited SMC proliferation (Stewart *et al.*, 1997), consistent with the reported effect of cAMP on cell proliferation in other cell types. Their selective effect on bronchoconstriction in SMC might be outweighed by adverse effects of the drugs on other immune cells, such as neutrophils, where the activation of PKA increases survival.

Glucocorticoids are a type of steroids that reduce swelling, and may be used in conjunction with mucolytics to reduce the build-up of mucus. However, inflammation was not suppressed by treatment with corticosteroids (Brown, 2007) perhaps because glucocorticoids are known to disrupt apoptotic signalling in neutrophils and thus prolong their survival (Liles *et al.*, 1995; Meagher *et al.*, 1996). Additionally, the involvement of glucocorticoid-regulated kinase 1 in the extension of neutrophil survival was demonstrated (Burgon *et al.*, 2014), which may additionally contribute to the occurrence of neutrophilia.

Selective PDE4 inhibitors were proposed as a novel therapeutical strategy in COPD, as they previously increased anti-inflammatory gene expression (Vignola, 2004; Wang, Cui, 2006; Brown, 2007; Crilly *et al.*, 2011). However, neutrophils secrete a wide range of cytokines, with pro-inflammatory mediators in addition to anti-inflammatory effectors (Tecchio *et al.*, 2014). The gastrointestinal side-effects of PDE4 inhibitors limited the dosing, and thus the efficacy of the drug (Brown, 2007). Likewise, low rolipram concentrations were unable to extend PMN survival to a great extent in the present study. However, as PDE4 inhibitors are also used to increase cAMP levels, they may contribute to neutrophilic inflammation by modulating neutrophil lifespan. Taken together most current therapeutic options may increase neutrophil presence through the prolongation of their survival, which may be disastrous in regards to the undesirable effects of neutrophil accumulation in the COPD lung, and novel survival specific targets in neutrophils are required to complement the current treatments. Taken together, this indicates that current treatments in COPD may be inadequate or even counteractive in the treatment of neutrophilia, as both corticosteroids and PDE inhibitors increase PMN survival (Bjornson *et al.*, 1985; Parkkonen *et al.*, 2008).

In regards to the findings of this thesis, it is proposed here that agents that increase cAMP may have beneficial effects on other parts of the disease, such as bronchoconstriction and potentially inflammatory gene expression. However, they may also exert detrimental effects by delaying neutrophil apoptosis. Therefore, it is proposed here that a targeted therapy to complement the current treatments may be necessary to control the detrimental effects induced by neutrophilia

and could involve increasing neutrophil apoptosis. This may be achieved by selective inhibitors of NR4A2, as the evidence of this study points towards a pro-survival effect of this molecule subtype. Moreover, we showed here that NR4A2 expression becomes increased by elevations in cAMP, as well as treatment with the inflammatory stimuli LPS and PGE2, suggesting that NR4A2 expression in the COPD lung may be greatly increased and may thus contribute to increased neutrophil survival and neutrophilia. Thus, the use of selective NR4A2 inhibitors may be useful in the treatment of excessive neutrophilia in chronic inflammation of COPD. Overall, NR4A2 might be a useful therapeutic strategy in the treatment of neutrophilia, as it appears to be invariably linked to PKA survival and can also be engaged by PGE2/EP2 signalling (Holla *et al.*, 2006; Shigeishi *et al.*, 2011). This might thus provide a target to compensate for the lack of neutrophil specific treatments in COPD, and complement the existing therapeutical strategies.

6.4. Limitations of the Model Systems.

Neutrophil lifespan is controlled by constitutive apoptosis. This element of neutrophil biology poses a great difficulty and can induce variability into the results obtained with neutrophils. Rates of basal apoptosis may vary in PMN, possibly depending on their age at extraction from whole blood, the general effect of a potential cytokine-withdrawal phenotype after the extraction from the blood and sensitivity to reagents used in the extraction procedure. Therefore, the culturing medium contains a percentage of serum, aiming to replicate the physiological conditions in whole blood and to minimise variation. Whereas differences in cell death rates are not a major obstacle for the research into many cellular functions, it represents a great difficulty for the investigation of cell survival signalling, as it hampers the distinction between relevant effects and random variation. Thus, a great amount of consistency is required in the scientific method used to minimise random variation.

Pharmacological modulators are widely used in research, but may introduce artefactual cytotoxicity that may be misinterpreted for physiologically relevant, reagent-specific effects. In this study, the usefulness of the pharmacological modulators was limited by various factors, as follows: 1) the specificity of selective agonists and antagonists was lost at increasing reagent concentrations, 2) high antagonist concentrations were required to prevent competitive replacement by the agonist, 3) comparisons between reagents were hampered by differences in their specific stability towards metabolism and 4) cell permeability.

Modulating gene expression levels by RNAi is therefore a common strategy used to circumvent the limitations listed above. However, PMN are genetically intractable, which requires the use of an alternative neutrophil model. In this study, mNØ were used here for this purpose. However, the investigation of mNØ survival pathways through selective gene expression knockdown was complicated by the lag-period between knockdown and cell differentiation, as

the proposed survival mediator was found to be also crucial to cell survival during the course of the differentiation.

6.5 Future Work

Here, it was demonstrated that PGE2 enhances survival through the action of PKA. PGE2-induced survival in PMN was slightly improved through pretreatment with LPS, which correlated with the upregulation of EP2 and EP4 receptor mRNA (and protein) expression. If the effect of LPS on PGE2 survival in PMN is a direct consequence of enhanced EP receptor expression, the influence of LPS may thus be even greater in COPD patient PMN, as LPS induced a higher level of EP2 receptor expression. Taken together with the reported increase of PGE2 expression in the COPD lung, this may potentially contribute to the neutrophilia observed in the condition. However, PGE2 furthermore controlled EP2 and EP4 expression levels by a negative feedback loop, which may serve as a regulatory mechanism of LPS-induced PKA survival *in vivo*. However, the relevance of this finding in an inflammatory setting *in vivo* would still need to be further elucidated.

Moreover, a further detailed investigation of the temporal regulation of EP2 and EP4 protein translation in healthy control and COPD PMN is warranted to substantiate the influence of LPS on PKA-dependent neutrophil survival, and the time point of the maximal biological response. Additionally, the molecular mechanisms involved in EP receptor expression regulation by LPS may elucidate the complex interplay of LPS and PGE2 PMN survival regulation.

The pro-survival influence of monocytes on PMN survival (Sabroe *et al.*, 2002a) was substantiated in this study, and it was suggested that monocyte-derived PGE2 secretion may induce PKA-dependent survival in PMN, as a crucial step in the sustenance of the inflammatory process. In particular, it needs to be tested, whether the enhanced survival in presence of PBMC may dependent on increases in PGE2 expression, potentially through previous exposure to inflammatory stimuli in the host. Interestingly, LPS is known to induce Cox-2 derived PGE2 secretion by monocytes, which might furthermore enhance neutrophil survival in an inflammatory setting. Thus, the basal presence of extracellular PGE2 in the culture medium and its influence on variability of basal PKA-dependence needs to be further elucidated. Additionally, monocyte-derived PGE2 secretion in healthy control or COPD blood may differ and thus influence constitutive rates of PMN survival and the sensitivity of their functional responses. In this context, no difference in EP2 and EP4 receptor expression was observed in PMN from healthy control and COPD patients. This furthermore substantiated current models in COPD, where neutrophils are considered as inflammatory mediators with no genetic abnormalities that are responsive to alterations in cytokine secretion by other immune cells.

Here, NR4A2 expression was PKA-dependently induced by PGE2. Although the tools for pharmacological modulation of NR4A2 are limited, an experimental agonist induced

survival, and similarly, *NR4A2* knockdown was associated with reduced cell numbers during differentiation and increased apoptosis in mature mNØ. Pro-survival effects of PGE2/PKA-mediated *NR4A2* expression have previously been described in the context of cancer (Shigeishi *et al.*, 2011). To substantiate the impact of PGE2-induced *NR4A2* expression in inflammation, the overexpressing of the nuclear receptor in the context of inflammation would be advantageous to be able to examine potential changes in cytokine expression or ROS secretion and explore, whether the effects of NR4A2 are selective towards PMN survival. This would enable the exploration of novel NR4A2 agonists in drug-development to complement the existing therapies.

Previously, a negative feedback loop of PGE2 on the regulation of its receptor expression was proposed. The role of NR4A2 in the regulation of EP2 and EP4 receptor expression by PGE2 would shed further light on the downstream effects of PGE2. In particular, to elucidate the complex interplay of LPS and PGE2 signalling, a detailed assay of the temporal events in LPS-induced survival upon PKA-inhibition, and the influence of EP receptor upregulation and *NR4A2* expression would be beneficial.

This may be complemented by a study on EP2 and EP4 localisation, as EP4 receptors have been found to be internalised, thus regulating PGE2 signalling (Chi *et al.*, 2014) and were functional, when found in the perinuclear space (Bhattacharya et al, 1999). Their granular appearance indicated that they may have localised effects, and thus the regulation of PKA localisation and function by AKAPs and their involvement in PMN survival would be interesting in this context. Moreover, NR4A2 and NR4A3 have previously been observed localised to the mitochondria, which might there exert an effect on local Cox-2 derived PGE2 expression.

In this study, PGE2 induced transient survival that was lost at 20 hours, which may be due to a loss of receptor responsiveness, may also depend on the metabolic degradation of PGE2. Interestingly, a substantial amount of research on the effect of PGE2 in neutrophils has been undertaken at late time points. Therefore, the degradation of PGE2 to metabolic products, such as 15-keto PGE2, and their involvement in late PMN survival may be of interest for the investigation of neutrophil survival *in vitro*, as the effects that were previously attributed to PGE2 at late time points, might not be specific to PGE2, but its metabolic products. PGE2 is expected to be continuously produced in inflammation *in vivo*, and may indicate that the short PMN lifespan *in vitro* is induced by a cytokine-withdrawal phenotype through the isolation of the cells from the blood. Thus, future work following from this observation may involve a meta-analysis of previous studies on PMN function.

6.6 Summary

In the course of this research project, the following specific questions were addressed:

1) Does PGE2 induce PKA dependent survival in PMN?
2) Does the selective stimulation of prostaglandin receptors attenuate neutrophil survival?
3) Do inflammatory mediators present in the context of COPD perpetuate PGE2-induced survival?
4) Are nuclear receptors NR4A2 and NR4A3 molecular targets that may be used to specifically block PGE2-induced PKA survival in neutrophils?

Thus, the scope of this dissertation was to investigate the interplay of PGE2 and cAMP/PKA signalling in the context of COPD and their influence of neutrophil survival, as well as to elucidate potential roles for NR4A2 and NR4A3 as novel pathway components in PKA signalling and neutrophil survival, respectively. The answers to the questions outlined above are of major clinical importance, as neutrophilia is an aspect of COPD pathology that is often dismissed, as having a minor impact on inflammation. Partially, this is due to the historic development of the area, because neutrophils typically have a very short lifespan, and thus it was often theorised that they can only exert a minimal impact on inflammation. However, it is now well established that neutrophil lifespan largely exceeds the initially reported one and can also be further prolonged at inflammatory sites. In this study it was thus determined that:

1) PKA is a potent pro-survival agent in PGE2-induced survival in PMN.
2) An EP2 receptor phenotype was determined for the pro-survival effects of PGE2 in PMN.
3) The COPD-phenotype inducing agent LPS can increase EP2 and EP4 receptor expression, augmenting the functional influence of PGE2 on PMN survival.
4) NR4A2 and NR4A3 nuclear receptors are implicated in PKA-dependent PGE2 signalling. Moreover, NR4A2 has crucial roles in neutrophil development, potentially through its pro-survival effect.

To conclude, it was demonstrated here that PKA has an essential role in neutrophil survival and that the increased presence of PGE2 and LPS in the COPD lung may contribute to the phenotype of neutrophilia. Thus, the selective targeting of PKA pathway components, such as NR4A2, may be explored as a therapeutic strategy to relieve the detrimental symptoms induced by neutrophil accumulation in inflammatory diseases, such as COPD.

CHAPTER 7. BIBLIOGRAPHY

Key References

- Profita *et al.* (2010): Physiological concentrations of PGE2 are increased in COPD smokers.
- Ottonello *et al.* (1998): PGE2-mediated neutrophil survival is PKA-dependent.
- Crean *et al.* (2015): NR4A2 and NR4A3 are cooperatively induced by LPS and PKA signalling in monocytes.
- Wang *et al.* (2006): Description of an experimental strategy for conditional immortilisation of bone-marrow derived neutrophil progenitors and their subsequent differentiation into neutrophils
- McDonald *et al.* (2011): Description of the genetic modification of Hoxb8-immortalised neutrophil progenitors
- Kirschnek *et al.* (2011): First publication, where Hoxb8 neutrophils were used for the investigation of neutrophil apoptosis. Anti-apoptotic Bcl-2 proteins were downregulated before constitutive apoptosis, and apoptosis could only be prevented by loss of both BH3-only proteins, Bim and Noxa

Reference List

Aarbiou, J., Rabe, K. F., Hiemstra, P. S. (2002). Role of defensins in inflammatory lung disease. *Ann Med.* 34: 96-101.

Aaron, S. D., Angel, J. B., Lunau, M., Wright, K., Fex, C., Le Saux, N., Dales, R. E. (2001). Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 163:349–55.

Abegunde, D.O., Mathers, C.D., Taghreed, A., Ortegon, M., Strong, K. (2007). The burden and costs of chronic diseases in low-income and middle-income countries. *Lancet.* 370:1929–38.

Abramson, M. J., Koplin, J., Hoy, R., Dharmage, S. C. (2015). Population-wide preventive interventions for reducing the burden of chronic respiratory disease. *Int J Tuberc Lung Dis*. 19(9):1007-18.

Af Forselles, K. J., Root, J., Clarke, T., Davey, D., Aughton, K., Dack, K., Pullen, N. (2011). In vitro and in vivo characterization of PF-04418948, a novel, potent and selective prostaglandin EP_2 receptor antagonist. Br J Pharmacol. 164(7):1847-56.

Akaogi, J., Yamada, H., Kuroda, Y., Nacionales, D. C., Reeves, W. H., Satoh, M. (2004). Prostaglandin E2 receptors EP2 and EP4 are up-regulated in peritoneal macrophages and joints of pristane-treated mice and modulate TNF- and IL-6 production. *Journal of Leukocyte Biology.* 76(1):227-36.

Akaogi, J., Nozaki, T., Satoh, M., Yamada, H. (2006). Role of PGE2 and EP receptors in the pathogenesis of rheumatoid arthritis and as a novel therapeutic strategy. *Endocr Metab Immune Disord Drug Targets.* 6(4):383-94.

Alba, G., El Bekay, R., Álvarez-Maqueda, M., Chacón, P., Vega, A., Monteseirín, J., Santa María, C., Pintado, E., Bedoya, F. J., Bartrons, R., Sobrino, F. (2004). Stimulators of AMP-activated protein kinase inhibit the respiratory burst in human neutrophils. *FEBS Lett.* 573(1-3):219-25.

Alfaidi, M., Wilson, H., Daigneault, M., Burnett, A., Ridger, V., Chamberlain, J., Francis, S. (2015). Neutrophil Elastase promotes Interleukin-1β secretion from Human Coronary Endothelium. *J Biol Chem*. jbc.M115.659029. [Epub ahead of print]

Allport, J. R., Ding, H. T., Ager, A., Steeber, D. A., Tedder, T. F., Luscinskas, F. W. (1997). L-selectin shedding does not regulate human neutrophil attachment, rolling, or transmigration across human vascular endothelium *in vitro*. *The Journal of Immunology*. 158:4365-4372.

Almansa, R., Socias, L., Sanchez-Garcia, M., Martín-Loeches, I., del Olmo, M., Andaluz-Ojeda, D., Bobillo, F., Rico, L., Herrero, A., Roig, V., San-Jose, C. A., Rosich, S., Barbado, J., Disdier, C., de Lejarazu, R. O., Gallegos, M. C., Fernandez, V., Bermejo-Martin, J. F.

(2012). Critical COPD respiratory illness is linked to increased transcriptomic activity of neutrophil proteases genes. *BMC Res. Notes*. 5:401.

Altstaedt, J., Kirchner, H., Rink, L. (1996). Cytokine production of neutrophils is limited to interleukin-8. *Immunology*. 89(4):563–568.

Amann, R., Egger, T., Schuligoi, R., Heinemann, A., Peskar, B. A. (2001). Sodium salicylate enhances the expression of cyclooxygenase-2 in endotoxin-stimulated human mononuclear cells. *Eur J Pharmacol*. 433(1):129-34.

Amieux, P. S., Cummings, D. E., Motamed, K., Brandon, E. P., Wailes, L. A., Le, K., Idzerda, R. L., McKnight, G. S. (1997). Compensatory regulation of RIalpha protein levels in protein kinase A mutant mice. *J Biol Chem*. 272(7):3993-8.

Andina, N., Conus, S., Schneider, E.M., Fey, M.F. & Simon, H.U. (2009) Induction of Bim limits cytokine-mediated prolonged survival of neutrophils. *Cell Death and Differentiation.* 16, 1248-1255.

Andreakos, E., Sacre, S. M., Smith, C., Lundberg, A., Kiriakidis, S., Stonehouse, T., Monaco, C., Feldmann, M., Foxwell, B. M. (2004). Distinct pathways of LPS-induced NF-kappa B activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. *Blood*. 103(6):2229-37.

Appelt, U., Sheriff, A., Gaipl, U. S., Kalden, J. R., Voll, R. E., Herrmann1, M. (2005). Viable, apoptotic and necrotic monocytes expose phosphatidylserine: cooperative binding of the ligand Annexin V to dying but not viable cells and implications for PS-dependent clearance. *Cell Death and Differentiation*. 12, 194–196.

Appleton, S., Poole, P., Smith, B., Veale, A., Bara, A. (2002). Long-acting beta2-agonists for chronic obstructive pulmonary disease patients with poorly reversible airflow limitation. *Cochrane Database Syst Rev*. (3):CD001104.

Appleton, S., Poole, P., Smith, B., Veale, A., Lasserson, T. J., Chan, M. M. (2006). Long-acting β2-agonists for poorly reversible chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*. 3:CD001104.

Arai, N., Kondo, M., Izumo, T., Tamaoki, J., Nagai, A. (2010). Inhibition of neutrophil elastase-induced goblet cell metaplasia by tiotropium in mice. *European Respiratory Journal*. 35(5): 1164–1171.

Arakawa, T., Laneuville, O., Miller, C. A., Lakkides, K. M., Wingerd, B. A., DeWitt, D. L., Smith, W. L. (1996). Prostanoid Receptors of Murine NIH 3T3 and RAW 264.7 Cells. Structure and Expression of the Murine Prostaglandin EP4 Receptor Gene. *J. Biol. Chem*. 271, 29569–29575.

Armstrong, R. A. (1995). Investigation of the inhibitory effects of PGE2 and selective EP agonists on chemotaxis of human neutrophils. *Br J Pharmacol*. 116(7):2903-8.

Armstrong, R. A., Talpain, E. (1994). Comparison of the prostaglandin E (EP) receptor of human neutrophils and HL-60 cells differentiated with DMSO. *Prostaglandins*. 48(4):221-34.

Arosh, J. A., Banu, S. K., Chapdelaine, P., Emond, V., Kim, J. J., MacLaren, L. A., Fortier, M. A. (2003). Molecular cloning and characterization of bovine prostaglandin E2 receptors EP2 and EP4: expression and regulation in endometrium and myometrium during the estrous cycle and early pregnancy. *Endocrinology.* 144(7):3076-91.

Assad, N. A., Balmes, J., Mehta, S., Cheema, U., Sood, A. (2015). Chronic obstructive pulmonary disease secondary to household air pollution. *Semin Respir Crit Care Med*. 36(3):408-21.

Atkinson, J. J., Senior, R. M. (2003). Matrix metalloproteinase-9 in lung remodeling. *Am. J. Respir. Cell Mol. Biol*. 28(1):12–24.

Au, B. T., Teixeira, M. M., Collins, P. D., Williams, T. J. (1998). Effect of PDE4 inhibitors on zymosan-induced IL-8 release from human neutrophils: synergism with prostanoids and salbutamol. *Br J Pharmacol.* 123:1260-1266.

Avdi, N.J., Nick,J.A., Whitlock, B.B., *et al.* (2001). Tumor necrosis factor-alpha activation of the c-Jun N-terminal kinase pathway in human neutrophils. Integrin involvement in a pathway leading from cytoplasmic tyrosine kinases apoptosis. *J Biol Chem.* 276:2189-2199.

Babu, K. S., Kastelik, J. A., Morjaria, J. B. (2014). Inhaled corticosteroids in chronic obstructive pulmonary disease: a pro–con perspective. *Br J Clin Pharmacol*. 78(2): 282–300.

Bae, Y. S., Lee, J. H., Choi, S. H., Kim, S., Almazan, F., Witztum, J. L., Miller, Y. I. (2009). Macrophages generate reactive oxygen species in response to minimally oxidized low-density lipoprotein: toll-like receptor 4- and spleen tyrosine kinase-dependent activation of NADPH oxidase 2. *Circ Res*. 104(2):210-8.

Bae, H.-B., Zmijewski, J. W., Deshane, J. S., Tadie, J.-M., Chaplin, D. D., Takashima, S., Abraham, E. (2011). AMP-activated protein kinase enhances the phagocytic ability of macrophages and neutrophils. *FASEB J*. 25(12):4358-68.

Baines, K. J., Simpson, J. L., Gibson, P. G. (2011). Innate Immune Responses Are Increased in Chronic Obstructive Pulmonary Disease. *PLOS One*. 6(3):e18426.

Bainton, D. F., Ullyot, J. L., Farquhar, M. G: (1971).The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J Exp Med.* 134:907-934.

Baker, K. D., Shewchuk, L. M., Kozlova, T., Makishima, M., Hassell, A., Wisely, B., Caravella, J. A., Lambert, M. H., Reinking, J. L., Krause, H., Thummel, C. S., Willson, T. M., Mangelsdorf, D. J. (2003). The Drosophila orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. *Cell*. 113(6):731-42.

Balaban, R. S., Nemoto, S., Finkel, T. (2005). Mitochondria, oxidants, and aging. *Cell*. 120:483–495.

Balbi, B., Bason, C., Balleari, E., Fiasella, F., Pesci, A., Ghio, R., Fabiano, F. (1997). Increased bronchoalveolar granulocytes and granulocyte/macrophage colony-stimulating factor during exacerbations of chronic bronchitis. *Eur Respir J*. 10:846-850.

Banič, B., Nipič, D., Suput, D., Milisav, I. (2011). DMSO modulates the pathway of apoptosis triggering. *Cell Mol Biol Lett.* 16(2):328-41.

Barletta, K. E., Ley, K., Mehrad, B. (2012). Regulation of Neutrophil Function by Adenosine. *Arterioscler Thromb Vasc Biol*. 32:856-864.

Barnes, P. J. (1993). Beta-adrenoceptors on smooth muscle, nerves and inflammatory cells. *Life Sci.* 52(26):2101-9.

Barnes, P. J. (2003). New concepts in chronic obstructive pulmonary disease. *Annu. Rev. Med*. 54(1):113–129.

Barnes, P. J. (2006). How corticosteroids control inflammation: Quintiles Prize Lecture 2005. *British Journal of Pharmacology*. 148: 245–254.

Barnes, P. J. (2008). Immunology of asthma and chronic obstructive pulmonary disease. *Nature Reviews Immunology.* 8:183-192.

Barnes, P. J. (2013). Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease. *J Allergy Clin Immunol*. 131:636-45.

Barnett, C. C. Jr., Moore, E. E., Partrick, D. A., Silliman, C. C. (1997). Beta-adrenergic stimulation down-regulates neutrophil priming for superoxide generation, but not elastase release. *J Surg Res*. 70(2):166-70.

Baselt, R. (2011). *Disposition of Toxic Drugs and Chemicals in Man (9th ed.).* Seal Beach, CA: Biomedical Publications. 236–9.

Bassini-Cameron, A., Sweet, E., Bottino, A., Bittar, C., Veiga, C., Cameron, L.-C. (2007). Effect of caffeine supplementation on haematological and biochemical variables in elite soccer players under physical stress conditions. *Br J Sports Med*. 41(8): 523–530.

Basu, S., Hodgson, G., Katz, M., Dunn, A. R. (2002). Evaluation of role of G-CSF in the production, survival, and release of neutrophils from bone marrow into circulation. *Blood.* 100:854-861.

Bäumer, W., Hoppmann, J., Rundfeldt, C., Kietzmann, M. (2007). Highly selective phosphodiesterase 4 inhibitors for the treatment of allergic skin diseases and psoriasis. *Inflamm Allergy Drug Targets*. 6(1):17-26.

Bazzoni, F., Cassatella, M. A., Rossi, F., Ceska, M., Dewald, B., Baggiolini, M. (1991). Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. *J Exp Med.* 173: 771-774.

Beeh, K. M., Beier, J., Kornmann, O., Micke, P., Buhl, R. (2001). Sputum levels of metalloproteinase-9 and tissue inhibitor of metalloproteinase-1, and their ratio correlate with airway obstruction in lung transplant recipients: relation to tumor necrosis factor-a and interleukin-10. *J. Heart Lung Transplant.* 20(11):1144–1151.

Beeh, K. M., Kornmann, O., Buhl, R., Culpitt, S. V., Giembycz, M. A., Barnes, P. J. (2003). Neutrophil chemotactic activity of sputum from patients with COPD: role of interleukin 8 and leukotriene B4. *Chest*. 123:1240-1247.

Beghe, B., Rabe, K. F., Fabbri, L. M. (2013). Phosphodiesterase-4 inhibitor therapy for lung diseases. *Am. J. Respir. Crit Care Med*. 188(3):271–278.

Belinsky, F., Nativ, N., Stelzer, G., Zimmerman, S., Iny Stein, T., Safran, M., Lancet, D. (2015). PathCards: multi-source consolidation of human biological pathways. *Database*. 2015:bav006. Supplementary information accessed from: www.genecards.org on 11th of September 2015.

Belmokhtar, C.A., Hillion, J. & Segal-Bendirdjian, E. (2001) Staurosporine induces apoptosis through both caspase-dependent and caspase-independent mechanisms. *Oncogene.* 20 (26):3354-3362.

Betsuyaku, T., Nishimura, M., Takeyabu, K., Tanino, M., Venge, P., Xu, S., Kawakami, Y. (1999). Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Am J Respir Crit Care Med*. 159(6):1985-91.

Bhattacharya, M., Peri, K., Ribeiro-da-Silva, A., Almazan, G., Shichi, H., Hou, X., Varma, D. R., Chemtob, S. (1999). Localization of functional prostaglandin E2 receptors EP3 and EP4 in the nuclear envelope. *J Biol Chem*. 274(22):15719-24.

Bianchi, M. E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. Journal of Leukocyte Biology. *Journal of Leukocyte Biology*. 81:1-5. Review.

Bianchi, S. M., Dockrell, D. H., Renshaw, S. A., Sabroe, I., Whyte, M. K. (2006). Granulocyte apoptosis in the pathogenesis and resolution of lung disease. *Clin Sci (Lond)*. 110(3):293-304.

Bichet, D.G. (2006) Nephrogenic diabetes insipidus. *Nephrologie & therapeutique*, 2, 387-404.

Bicknell, S., van Eeden, S., Hayashi, S., Hards, J., English, D., Hogg, J. C. (1994). A non-radioisotopic method for tracing neutrophils *In vivo* using 5′-bromo-2′-deoxyuridine. *Am. J. Respir. Cell Mol. Biol*. 10:16–23.

Biernacki, W. A., Kharitonov, S. A., Barnes, P. J. (2003). Increased leukotriene B4 and 8-isoprostane in exhaled breath condensate of patients with exacerbations of COPD. *Thorax*. 58:294–8.

Biffl, W. L., Moore, E. E., Moore, F. A., Barnett, C. C. Jr., Carl, V. S., Peterson, V. N. (1996). Interleukin-6 delays neutrophil apoptosis. *Arch Surg*. 131:24–29.

Birrell, M. A., Patel, H. J., McCluskie, K., Wong, S., Leonard, T., Yacoub, M. H., Belvisi, M. G. (2004). PPAR-gamma agonists as therapy for diseases involving airway neutrophilia. *Eur Respir J.* 24(1):18-23.

Birrell, M. A., Maher, S. A., Dekkak, B., Jones, V., Wong, S., Brook, P., Belvisi, M. G. (2015). Anti-inflammatory effects of PGE2 in the lung: role of the EP4 receptor subtype. *Thorax*. 70(8):740-7.

Bjomson, B. H., Harvey, J. M., Rose, L. (1985). Differential Effect of Hydrocortisone on Eosinophil and Neutrophil Proliferation. *J. Clin. Invest. The American Society for Clinical Investigation*. 76: 924-929.

Blidberg, K., Palmberg, L., Dahlén, B., Lantz, A. S., Larsson, K. (2012). Increased neutrophil migration in smokers with or without chronic obstructive pulmonary disease. *Respirology*. 17(5):854–860.

Blidberg, K., Palmberg, L., Dahlén, B., Lantz, A. S., Larsson, K. (2012). Chemokine release by neutrophils in chronic obstructive pulmonary disease. *Innate Immun*. 18(3):503–510.

Boneberg, E. M., Hartung, T. (2002). Molecular aspects of anti-inflammatory action of G-CSF. *Inflamm Res*. 51(3):119-28.

Boniface, K., Bak-Jensen, K. S., Li, Y., Blumenschein, W. M., McGeachy, M. J., McClanahan, T. K., McKenzie, B. S., Kastelein, R. A., Cua, D. J., de Waal Malefyt, R. (2009).

Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *J Exp Med*. 206(3):535–548.

Bonneau, O., Wyss, D., Ferretti, S., Blaydon, C., Stevenson, C. S., Trifilieff, A. (2006). Effect of adenosine A2A receptor activation in murine models of respiratory disorders. *Am J Physiol Lung Cell Mol Physiol.* 290:L1036–1043.

Bonta, P. I., van Tiel, C. M., Vos, M., Pols, T. W., van Thienen, J. V., Ferreira, V., Arkenbout, E. K., Seppen, J., Spek, C. A., van der Poll, T., Pannekoek, H., de Vries, C. J. (2006). Nuclear Receptors Nur77, Nurr1, and NOR-1 Expressed in Atherosclerotic Lesion Macrophages Reduce Lipid Loading and Inflammatory Responses. *Arterioscler Thromb Vasc Biol*. 26(10):2288-94.

Borchers, M. T., Carty, M. P., Leikauf, G. D. (1999). Regulation of human airway mucins by acrolein and inflammatory mediators. *Am J Physiol*. 276:L549—L555.

Bordon, Y. (2011). Innate immunity: Neutrophil U-turn fans the flames. *Nature Reviews Immunology*. 11:498. Review.

Boschetto, P., Beghé, B., Fabbri, L. M., Ceconi, C. (2012). Link between chronic obstructive pulmonary disease and coronary artery disease: implication for clinical practice. *Respirology*. 17(3):422-31.

Boysen, P., Eide, D.M. & Storset, A.K. (2011) Natural killer cells in free-living Mus musculus have a primed phenotype. *Molecular Ecology*, 20, 5103-5110.

Brach, M., deVos, S., Gruss, H., Herrmann, F. (1992). Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death. *Blood*. 80:2920–2924.

Bradfield, P. F., Scheiermann, C., Nourshargh, S., Ody, C., Luscinskas, F. W., Rainger, G. E. *et al*. (2007). JAM-C regulates unidirectional monocyte transendothelial migration in inflammation. *Blood*. 110: 2545-2555.

Brandon, E. P., Idzerda, R. L., McKnight, G. S. (1997). PKA isoforms, neural pathways, and behaviour: making the connection. *Curr Opin Neurobiol*. 7(3):397-403.

Brannigan, A. E., O'Connell, P. R., Hurley, H., O'Neill, A., Brady, H. R., Fitzpatrick, J. M., Watson, R. W. (2000). Neutrophil apoptosis is delayed in patients with inflammatory bowel disease. *Shock*. 13(5):361-6.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., Zychlinsky, A. (2004). Neutrophil Extracellular Traps Kill Bacteria. *Science*. 303(5663): 1532-1535.

Brinkman-Vand der Linden, E.C.M., Angata, T., Reynolds, S.A., Powell, L.D., Hedrick, S.M. & Varki, A. (2003) CD33/Siglec-3 binding specificity, expression pattern, and consequences of gene deletion in mice. *Molecular and Cellular Biology*, 23, 4199-4206.

Brock, T. G., McNish, R. W., Peters-Golden, M. (1999). Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E2. *J. Biol. Chem*. 274:11660-11666.

Brouckaert, G., Kalai, M., Krysko, D. V., Saelens, X., Vercammen, D., Ndlovu, M., Haegeman, G., D'Herde, K., Vandenabeele, P. (2004). Phagocytosis of Necrotic Cells by Macrophages Is Phosphatidylserine Dependent and Does Not Induce Inflammatory Cytokine Production. *Mol Biol Cell*. 15(3): 1089–1100.

Brown, R. A., Lever, R., Jones, N. A., Page, C. P. (2003). Effects of heparin and related molecules upon neutrophil aggregation and elastase release *in vitro*. Br J Pharmacol. 139(4): 845–853.

Brown, V., Elborn, J. S., Bradley, J., Ennis, M. (2009). Dysregulated apoptosis and NFκB expression in COPD subjects. *Respiratory Research*. 10:24.

Bucchioni, E., Kharitonov, S. A., Allegra, L., Barnes, P. J. (2003). High levels of interleukin-6 in the exhaled breath condensate of patients with COPD. *Respiratory Medicine*. 97, 1299–1302.

Buckley, C. D., Ross, E. A., McGettrick, H. M., Osborne, C., Haworth, O., Schmutz, C. *et al.* (2006). Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. *Journal of Leukocyte Biology*. 79, 303-311.

Burelout, C., Thibault, N., Harbour, D., Naccache, P. H., Bourgoin, S. G. (2007). The PGE2-induced inhibition of the PLD activation pathway stimulated by fMLP in human neutrophils is mediated by PKA at the PI3-Kgamma level. *Biochem Pharmacol*. 74(5):730-41.

Burgon, J., Robertson, A. L., Sadiku, P., Wang, X., Hooper-Greenhill, E., Prince, L. R., Walker, P., Hoggett, E. E., Ward, J. R., Farrow, S. N., Zuercher, W. J., Jeffrey, P., Savage, C. O., Ingham, P. W., Hurlstone, A. F., Whyte, M. K., Renshaw, S. A. (2014). Serum and glucocorticoid-regulated kinase 1 regulates neutrophil clearance during inflammation resolution. *J Immunol*. 192(4):1796-805.

Burnett, D., Chamba, A., Hill, S. L., Stockley, R. A. (1987). Neutrophils from subjects with chronic obstructive lung disease show enhanced chemotaxis and extracellular proteolysis. *Lancet*. 2(8567):1043-6.

Butt, E., 2015, 'Cyclic nucleotide analogs, as pharmacological tools for studying signalling pathways' in *Cyclic Nucleotide Signalling*, eds X Cheng, CRC Press, Boca Raton (FL), p. 21.

Calverley, P. M., Anderson, J. A., Celli, B., Ferguson, G. T., Jenkins, C., Jones, P. W., Yates, J. C., Vestbo, J. (2007). Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease. *N Engl J Med*. 356:775–789.

Cannon, M. J., Openshaw, P. J., Askonas, B. A. (1988). Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. *J Exp Med*. 168(3):1163–1168.

Caramori, G., Pandit, A., Papi, A. (2005). Is there a difference between chronic airway inflammation in chronic severe asthma and chronic obstructive pulmonary disease? *Curr. Opin. Allergy Clin. Immunol*. 5(1):77–83.

Carlson, C. R., Lygren, B., Berge, T., Hoshi, N., Wong, W., Taskén, K., Scott, J. D. (2006). Delineation of type I protein kinase A-selective signaling events using an RI anchoring disruptor. *J Biol Chem*. 281:21535-21545.

Carter, R. I., Ungurs, M. J., Mumford, R. A., Stockley, R. A. (2012). Aa-Val360: a marker of neutrophil elastase and COPD disease activity. *Eur. Respir. J*. 41(1):31–38.

Caserta, T.M., Smith, A.N., Gultice, A.D., Reedy, M.A. & Brown, T.L. (2003) Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. *Apoptosis*, 8, 345-352.

Casolaro, M. A., Bernaudin, J. F., Saltini, C., Ferrans, V. J., Crystal, R. G. (1988). Accumulation of Langerhans' cells on the epithelial surface of the lower respiratory tract in normal subjects in association with cigarette smoking. *Am Rev Respir Dis*. 137:406-411.

Cazzola, M., Imperatore, F., Salzillo, A., Di Perna, F., Calderaro, F., Imperatore, A., Matera, M. G. (1998). Cardiac effects of formoterol and salmeterol in patients suffering from COPD with pre-existing cardiac arrhythmias and hypoxemia. *Chest*. 114:411–415.

Cazzola, M., Calzetta, L., Matera, M. G. (2011). β2-adrenoceptor agonists: current and future direction. *Br J Pharmacol*. 163(1):4–17.

Chandramouli, A., Onyeagucha, B. C., Mercado-Pimentel, M. E., Stankova, L., Shahin, N. A., LaFleur, B. J., Heimark, R. L., Bhattacharyya, A. K., Nelson, M. A. (2012). MicroRNA-101 (miR-101) post-transcriptionally regulates the expression of EP4 receptor in colon cancers. *Cancer Biol Ther*. 13(3):175-83.

Chang, H. S., Jeon, K. W., Kim, Y. H., Chung, I. Y., Park, C. S. (2000). Role of cAMP-dependent pathway in eosinophil apoptosis and survival. *Cell. Immunol*. 203:29–38.

Chang, L. C., Madsen, S. A., Toelboell, T., Weber, P. S., Burton, J. L. (2004). Effect of glucocorticoids on Fas gene expression in bovine blood neutrophils. *J Endocrinol*. 183:569–683.

Chen, G., Goeddel, D. V. (2002). TNF-R1 signaling: a beautiful pathway. *Science.* 296(5573):1634–5. Review.

Chen, J. H., Perry, C. J., Tsui, Y.-C., Staron, M. M., Parish, I. A., Dominguez, C. X., Rosenberg, D. W., Kaech, S. M. (2015). Prostaglandin E2 and programmed cell death 1

signaling coordinately impair CTL function and survival during chronic viral infection. *Nature Medicine*. 21:327–334.

Cheng, L. E.-C., Chan, F. K.-M., Cado, D., Winoto, A. (1997). Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. *EMBO J*. 16:1865-1875.

Chi, Y., Suadicani, S. O., Schuster, V. L. (2014). Regulation of prostaglandin EP1 and EP4 receptor signaling by carrier-mediated ligand reuptake. *Pharmacol Res Perspect*. 2(5):e00051.

Chinetti, G., Griglio, S., Antonucci, M., Torra, I. P., Delerive, P., Majd, Z., Fruchart, J. C., Chapman, J., Najib, J., Staels, B. (1998). Activation of peroxisome proliferator-activated receptor α and γ induces apoptosis of human monocyte-derived macrophages. *J Biol Chem*. 273:25573–25580.

Chizzolini, C., Chicheportiche, R., Alvarez, M., de Rham, C., Roux-Lombard, P., Ferrari-Lacraz, S., Dayer, J. M. (2008). Prostaglandin E2 synergistically with interleukin-23 favors human Th17 expansion. *Blood*. 112(9):3696–3703.

Cho, H., Hamza, A., Zhan, C. G., Tai, H. H. (2005). Key NAD+-binding residues in human 15-hydroxyprostaglandin dehydrogenase. *Archives of biochemistry and biophysics*. 433(2):447-53.

Cho, H., Huang, L., Hamza, A., Gao, D., Zhan, C. G., Tai, H. H. (2006). Role of glutamine 148 of human 15-hydroxyprostaglandin dehydrogenase in catalytic oxidation of prostaglandin E2. *Bioorg Med Chem*. 14(19):6486-91.

Chochung, Y.S., Clair, T., Tagliaferri, P., Ally, S., Katsaros, D., Tortora, G., Neckers, L., Avery, T.L., Crabtree, G.W. & Robins, R.K. (1989) Site-selective cyclic-AMP analogs as new biological tools in growth-control, differentiation, and proto-oncogene regulation. *Cancer Investigation*, 7, 161-177.

Chomczynski, P., Mackey, K. (1995). Substitution of chloroform by bromo-chloropropane in the single-step method of RNA isolation. *Anal Biochem*. 225(1):163-4.

Chubb, A. J., Fitzgerald, D. J., Nolan, K. B., Moman, E. (2006). The productive conformation of prostaglandin G2 at the peroxidase site of prostaglandin endoperoxide H synthase: docking, molecular dynamics, and site-directed mutagenesis studies. *Biochemistry*. 45(3):811-20.

Codina, A., Benoit, G., Gooch, J. T., Neuhaus, D., Perlmann, T., Schwabe, J. W. (2004). Identification of a novel co-regulator interaction surface on the ligand binding domain of Nurr1 using NMR footprinting. *J Biol Chem*. 279(51):53338–53345.

Colamussi, M. L., White, M. R., Crouch, E., Hartshorn, K. L. (1999). Influenza A virus accelerates neutrophil apoptosis and markedly potentiates apoptotic effects of bacteria. *Blood*. 93:2395-2403.

Coleman, R. A., Denyer, L. H., Sheldrick, R. L. G. (1985). The influence of protein binding on the potency of the prostanoid EP1-receptor blocking drug, AH6809. *Br. J. Pharmacol*. 86:203P.

Coleman, R. A., Sheldrick, R. L. (1989). Prostanoid-induced contraction of human bronchial smooth muscle is mediated by TP-receptors. *Br J Pharmacol*. 96(3):688-92.

Colotta, F., Re, F., Polentarutti, N., Sozzani, S., Mantovani, A. (1992). Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood*. 80:2012–2020.

Condliffe, A. M., Chilvers, E. R., Haslett, C., Dransfield, I. (1996). Priming differentially regulates neutrophil adhesion molecule expression/function. *Immunology*. 89(1):105-11.

Condliffe, A. M., Kitchen, E., Chilvers, E. R. (1998). Neutrophil priming: pathophysiological consequences and underlying mechanisms. *Clin Sci (Lond)*. 94(5):461-71.

Conway, R. M., Madigan, M. C., Billson, F. A., Penfold, P. L. (1998). Vincristine- and Cisplatin-induced Apoptosis in Human Retinoblastoma - Potentiation by Sodium Butyrate. *Europ. J. Cancer*. 34:1741 – 1748.

Corhay, J. L., Henket, M., Nguyen, D., Duysinx, B., Sele, J., Louis, R. (2009). Leukotriene B4 contributes to exhaled breath condensate and sputum neutrophil chemotaxis in COPD. *Chest*. 136(4):1047-54.

Corrigan, C. J., Napoli, R. L., Meng, Q., Fang, C., Wu, H., Tochiki, K., Reay, V., Lee, T. H., Ying, S. (2012). Reduced expression of the prostaglandin E2 receptor E-prostanoid 2 on bronchial mucosal leukocytes in patients with aspirin-sensitive asthma. *J Allergy Clin Immunol*. 129(6):1636-46.

Cowburn, A. S., Deighton, J., Walmsley, S. R., Chilvers, E. R. (2004). The survival effect of TNFa in human neutrophils is mediated via NF-kappa B-dependent IL-8 release. *Eur J Immunol*. 34:1733–1743.

Cowland, J.B. & Borregaard, N. (1999) Isolation of neutrophil precursors from bone marrow for biochemical and transcriptional analysis. *Journal of Immunological Methods*, 232, 191-200.

Coxon, A., Rieu, P., Barkalow, F. J., Askari, S., Sharpe, A. H., von Andrian, U. H. *et al.* (1996). A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. *Immunity*. 5:653-666.

Coxon, A., Tang, T., Mayadas, T. N. (1999). Cytokine-Activated Endothelial Cells Delay Neutrophil Apoptosis *in vitro* and *In vivo*: A Role for Granulocyte/Macrophage Colony-Stimulating Factor. *J Exp Med*. 190(7): 923–934.

Crean, D., Cummins, E. P., Bahar, B., Mohan, H., McMorrow, J. P., Murphy, E. P. (2015). Adenosine Modulates NR4A Orphan Nuclear Receptors To Attenuate Hyperinflammatory Responses in Monocytic Cells. *The Journal of Immunology*. 195(4): 1436-448.

Crilly, A., Robertson, S. E., Reilly, J. H., Gracie, J. A., Lai, W. Q., Leung, B. P., Life, P. F., McInnes, I. B. (2011). Phosphodiesterase 4 (PDE4) regulation of proinflammatory cytokine and chemokine release from rheumatoid synovial membrane. *Ann Rheum Dis*. 70(6):1130-7.

Cross, A., Moots, R. J., Edwards, S. W. (2008). The dual effects of TNFα on neutrophil apoptosis are mediated via differential effects on expression of Mcl-1 and Bfl-1. *Blood.* 111(2):878-884.

Cryer B, Feldman M. (1998). Cyclooxygenasse-1 and cyclooxygenase-2 selectivity of widely used nonsteroidal anti-inflammatory drugs. *Am J Med*. 104(5):413-421.

Cui, J., Chen, C., Lu, H., Sun, T., Shen, P. (2008). Two independent positive feedbacks and bistability in the Bcl-2 apoptotic switch. *PLoS ONE*. 3(1):e1469.

Cuisset, L., Tichonicky, L., Delpech, M. (1998). A Protein Phosphatase is Involved in the Inhibition of Histone Deacetylation by Sodium Butyrate. *Biochem. & Biophys. Res. Commun*. 246:760-764.

Culpitt, S. V., Nightingale, J. A., Barnes, P. J. (1999). Effect of high dose inhaled steroid on cells, cytokines and proteases in induced sputum in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 160:1635-1639.

Culpitt, S. V., Rogers, D. F., Shah, P., de Matos, C., Russell, R. E., Donnelly, L. E., Barnes, P. J. (2003). Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 167:24-31.

Daffern, P. J., Jagels, M. A., Hugli, T. E. (1999). Multiple epithelial cell–derived factors enhance neutrophil survival: regulation by glucocorticoids and tumor necrosis factor-a. *Am. J. Respir. Cell Mol. Biol*. 21:259–267.

Dagouassat, M., Gagliolo, J. M., Chrusciel, S., Bourin, M. C., Duprez, C., Caramelle, P., Boyer, L., Hue, S., Stern, J. B., Validire, P., Longrois, D., Norel, X., Dubois-Randé, J. L., Le Gouvello, S., Adnot, S., Boczkowski, J. (2013). The cyclooxygenase-2-prostaglandin E2 pathway maintains senescence of chronic obstructive pulmonary disease fibroblasts. *Am J Respir Crit Care Med*. 187(7):703-14.

Daldegan, M. B., Teixeira, M. M., Talvani, A. (2005). Concentration of CCL11, CXCL8 and TNF-alpha in sputum and plasma of patients undergoing asthma or chronic obstructive pulmonary disease exacerbation. *Braz J Med Biol Res*. 38(9):1359-65.

Damiano, V. V., Tsang, A., Kucich, U., Abrams, W. R., Rosenbloom, J., Kimbel, P., Fallahnejad, M., Weinbaum, G. (1986). Immunolocalization of elastase in human emphysematous lungs. *J Clin Invest.* 78(2):482–493.

D'Armiento, J. D. S., Okada, Y., Berg, R. A., Chada, K. (1992). Collagenase expression in the lungs of transgenic mice causes pulmonary emphysema. *Cell*. 71:955–961.

Darragh, J., Soloaga, A., Beardmore, V. A., Wingate, A. D., Wiggin, G. R., Peggie, M., Arthur, J. S. (2005). MSKs are required for the transcription of the nuclear orphan receptors Nur77, Nurr1 and Nor1 downstream of MAPK signalling. *Biochem J*. 390:749–759.

Davies-Cox, E. V., Laffafian, I., Hallett, M. B. (2001). Control of Ca2+ influx in human neutrophils by inositol 1,4,5-trisphosphate (IP3) binding: differential effects of micro-injected IP3 receptor antagonists. *Biochem J.* 355(Pt 1):139-43.

de Boer, W. I., Sont, J. K., van Schadewijk, A., Stolk, J., van Krieken, J. H., Hiemstra, P. S. (2000). Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD. *J Pathol*. 190(5):619-26.

de Jong, J. W., Belt-Gritter, B., Koeter, G. H., Postma, D. S. (1997). Peripheral blood lymphocyte cell subsets in subjects with chronic obstructive pulmonary disease: association with smoking, IgE and lung function. *Respir Med*. 91:67-76.

de Rooij, J., Zwartkruis, F. J. T., Verheijen, M. H. G., Cool, R. H., Nijman, S. M. B., Wittinghofer, A., Bos, J. L. (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*. 396:474-477.

de Serres, F. J., Blanco, I. (2012). Prevalence of α1-antitrypsin deficiency alleles PI*S and PI*Z worldwide and effective screening for each of the five phenotypic classes PI*MS, PI*MZ, PI*SS, PI*SZ, and PI*ZZ: a comprehensive review. *Ther Adv Respir Dis*. 6(5):277-95.

Degterev, A., Boyce, M., Yuan, J. (2003). A decade of caspases. *Oncogene*. 22, 8543–8567.

DeLeo, F. R. (2004). Modulation of phagocyte apoptosis by bacterial pathogens. *Apoptosis*. 9:399–413.

Dell'Acqua, M.L. (2003) *Subcellular Targeting of PKA through AKAPs: Conserved Anchoring and Unique Targeting Domains in Handbook of Cell Signaling*, Volume 2 (Bradshaw and Dennis Eds.), Elsevier Academic Press, San Diego, CA, Vol. 2:185, pp.377-381.

Desai, S., Ashby, B. (2001). Agonist-induced internalization and mitogen-activated protein kinase activation of the human prostaglandin EP4receptor. *FEBS Lett*. 501(2-3):156-60.

Deutsch, A. J., Angerer, H., Fuchs, T. E., Neumeister, P. (2012). The nuclear orphan receptors NR4A as therapeutic target in cancer therapy. *Anticancer Agents Med Chem*. 12(9):1001-14.

D'Hulst, A., Vermeulen, K. Y., Pauwels, R. A. (2002). Cigarette smoke exposure causes increase in pulmonary dendritic cells. *Am J Respir Crit Care Med*. 164:A604.

Di Marco, F., Milic-Emili, J., Boveri, B., Carlucci, P., Santus, P., Casanova, F., Cazzola, M., Centanni, S. (2003). Effect of inhaled bronchodilators on inspiratory capacity and dysnoea at rest in COPD. *Eur Respir J*. 21:86–94.

Di Stefano, A., Capelli, A., Lusuardi, M., Balbo, P., Vecchio, C., Maestrelli, P., Mapp, C. E., Fabbri, L. M., Donner, C. F., Saetta, M. (1998). Severity of airflow limitation is associated with severity of airway inflammation in smokers. *Am J Respir Crit Care Med*. 158(4):1277-1285.

Diaz, P. T., King, M. A., Pacht, E. R., Wewers, M. D., Gadek, J. E., Nagaraja, H. N., Drake, J., Clanton, T. L. (2000). Increased susceptibility to pulmonary emphysema among HIV-seropositive smokers. *Ann Intern Med*. 132(5):369–372.

Dibbert, B., Weber, M., Nikolaizik, W. H., Vogt, P., Schöni, M. H., Blaser, K., Simon, H.-U. (1999). Cytokine-mediated Bax deficiency and consequent delayed neutrophil apoptosis: A general mechanism to accumulate effector cells in inflammation. *Proc Natl Acad Sci U S A.* 96(23):13330–13335.

Dick, E. P., Prince, L. R., Prestwich, E. C., Renshaw, S. A., Whyte, M. K., Sabroe, I. (2009). Pathways regulating lipopolysaccharide-induced neutrophil survival revealed by lentiviral transduction of primary human neutrophils. *Immunology.* 127(2):249-55.

Dive, C., Gregory, C. D., Phipps, D. J., Evans, D. I., Milncr, A. E., Wyllie, A. H. (1992). Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim Biophys Acta.* 113(3):275-285.

Doeing, D.C., Borowicz, J.L. & Crockett, E.T. (2003) Gender dimorphism in differential peripheral blood leukocyte counts in mice using cardiac, tail, foot, and saphenous vein puncture methods. *BMC Clin. Pathol*. 3(1):3.

Domingo-Gonzalez, R., Martínez-Colón, G. J., Smith, A. J., Smith, C. K., Ballinger, M. N., Xia, M., Murray, S., Kaplan, M. J., Yanik, G. A., Moore, B. B. (2015). Prostaglandin E2 Inhibits Neutrophil Extracellular Trap Formation Post-stem Cell Transplant. Am J Respir Crit Care Med. [Epub ahead of print]

Drost, E. M., Macnee, W. (2002b). Potential role of IL8, platelet activating factor and TNF-alpha in the sequestration of neutrophils in the lung: effects on neutrophil deformability, adhesion receptor expression and chemotaxis. *Eur. J. Immunol.* 32: 393-4003.

Drost, E. M., Skwarski, K. M., Sauleda, J., Soler, N., Roca, J., Agusti, A., MacNee, W. (2005). Oxidative stress and airway inflammation in severe exacerbations of COPD. *Thorax*. 60(4):293-300.

Duarte, R. F., Frank, D. A. (2002). The Synergy Between Stem Cell Factor (SCF) and Granulocyte Colony-stimulating Factor (G-CSF): Molecular Basis and Clinical Relevance. *Leukemia & Lymphoma*. 43(6):1179-1187.

Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, Lipsky PE (1998). Cyclooxygenase in biology and disease. *FASEB J*. 12:1063–1073.

Dubois, C., Hengerer, B., Mattes. H. (2006). Identification of a potent agonist of the orphan nuclear receptor Nurr1. *ChemMedChem*. 1(9):955–958.

Dubravec, D. B., Spriggs, D. R., Mannick, J. A., Rodrick, M. L. (1990). Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha. *Proc Natl Acad Sci USA*. 87(17):6758-61.

Dux, R., Kindler-Röhrborn, A., Annas, M., Faustmann, P., Lennartz, K., Zimmermann, C. W. (1994). A standardized protocol for flow cytometric analysis of cells isolated from cerebrospinal fluid. *J Neurol Sci*. 121(1):74-8.

Edelman, A., Blumenthal, D. K., Krebsm, E. G. (1987). Protein serine/threonine kinases. Ann. Rev. Biochem. 56:567-613.

Edwards, S. W. (1994). *Biochemistry and physiology of the neutrophil*. London: Cambridge University Press.

Elliott, A. D., Ustione, A., Piston, D. W. (2015). Somatostatin and insulin mediate glucose-inhibited glucagon secretion in the pancreatic α-cell by lowering cAMP. *American Journal of Physiology - Endocrinology and Metabolism.* 308(2):E130-E143.

Eltboli, O., Bafadhel, M., Hollins, F., Wright, A., Hargadon, B., Kulkarni, N., Brightling, C. (2014). COPD exacerbation severity and frequency is associated with impaired macrophage efferocytosis of eosinophils. *BMC Pulm Med*. 14:112.

Ertel, W., Keel, M., Infanger, M., Ungethum, U., Steckholzer, U., Trentz, O. (1998). Circulating mediators in serum of injured patients with septic complications inhibit neutrophil apoptosis through up-regulation of protein-tyrosine phosphorylation. *J. Trauma*. 44:767–776.

Esther, C. R. Jr., Alexis, N. E., Clas, M. L., Lazarowski, E. R., Donaldson, S. H., Ribeiro, C. M., Moore, C. G., Davis, S. D., Boucher, R. C. (2008). Extracellular purines are biomarkers of neutrophilic airway inflammation. *Eur Respir J*. 31(5):949-56.

Fadeel, B., Ahlin, A., Henter, J. I., Orrenius, S., Hampton, M. B. (1998). Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species. *Blood*. 92(12):4808-18.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., Henson, P. M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol.* 148 (7): 2207-2216.

Fadok, V. A., Bratton, D. L., Rose, D. M., Pearson, A., Ezekewitz, R. A., Henson, P. M. (2000). A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature (Lond)*. 405:85-90.

Fadok, V. A., de Cathelineau, A., Daleke, D. L., Henson, P. M., Bratton, D. L. (2001). Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for

phagocytosis of apoptotic cells by macrophages and fibroblasts. *J. Biol. Chem*. 276,1071-1077.

Feldmann, H., Bugany, H., Mahner, F., Klenk, H. D., Drenckhahn, D., & Schnittler, H. J. (1996). Filovirus-induced endothelial leakage triggered by infected monocytes/macrophages. *The Journal of Virology.* 70: 2208-2214.

Ferreri, N. R., Sarr, T., Askenase, P. W., Ruddle, N. H. (1992). Molecular regulation of tumor necrosis factor-alpha and lymphotoxin production in T cells: Inhibition by prostaglandin E2. *J Biol Chem*. 267:9443.

Finkelstein, R., Fraser, R. S., Ghezzo, H., Cosio, M. G. (1995). Alveolar inflammation and its relation to emphysema in smokers. *Am J Respir Crit Care Med*. 152(5 Pt 1):1666-72.

Finlay, G. A., O'Driscoll, L. R., Russell, K. J., D'Arcy, E. M., Masterson, J. B., FitzGerald, M. X., O'Connor, C. M. (1997). Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. *Am J Respir Crit Care Med*. 156:240–247.

Finney-Hayward, T. K., Bahra, P., Li, S., Poll, C. T., Nicholson, A. G., Russell, R. E. K., Ford, P. A., Westwick, J., Fenwick, P. S., Barnes, P. J., Donnelly, L. E. (2009). Leukotriene B4 release by human lung macrophages via receptor- not voltage-operated Ca2 Channels. *Eur Respir J.* 33:1105–1112.

Fitzpatrick, F. A., Aguirre, R., Pike, J. E., Lincoln, F. H. (1980). The stability of 13,14-dihydro-15 keto-PGE2. *Prostaglandins*. 19:917–931.

Flaig, R., Greschik, H., Peluso-Iltis, C., Moras, D. (2005). Structural basis for the cell-specific activities of the NGFI-B and the Nurr1 ligand-binding domain. *J Biol Chem*. 280(19):19250-8.

Fogel-Petrovic, M., Long, J. A., Knight, D. A., Thompson, P. J., Upham, J. W. (2004). Activated human dendritic cells express inducible cyclo-oxygenase and synthesize prostaglandin E2 but not prostaglandin D2. *Immunology and Cell Biology*. 82:47–54.

Förstermann, U., Neufang, B. (1983). Elimination from the circulation of cats of 6-keto-prostaglandin E1 compared with prostaglandins E2 and I2. *J. Pharm. Pharmacol*. 35:724–728.

Fortier, M. A., Krishnaswamy, K., Danyod, G., Boucher-Kovalik, S., Chapdalaine, P. (2008). A postgenomic integrated view of prostaglandins in reproduction: implications for other body systems. *J Physiol Pharmacol.* 59 (1):65-89.Francis, S. H., Houslay, M. D., Conti, M. (2011). *Phosphodiesterases As Drug Targets.* Berlin-Heidelberg.

Franklin, W. A., Veve, R., Hirsch, F. R., Helfrich, B. A., Bunn, P. A. Jr. (2002). Epidermal growth factor receptor family in lung cancer and premalignancy. *Semin Oncol*. 29: 3-14.

Freeman, C. M., Martinez, F. J., Han, M. K., Washko, G. R. Jr., McCubbrey, A. L., Chensue, S. W., Arenberg, D. A., Meldrum, C. A., McCloskey, L., Curtis, J. L. (2013). Lung CD8+ T cells in COPD have increased expression of bacterial TLRs. *Respir. Res*. 14:13.

Friedewald, V. E., Bennett, J. S., Christo, J. P., Pool, J. L., Scheiman, J. M., Simon, L. S., Strand, V., White, W. B., Williams, G. W., Roberts, W. C. (2010). AJC Editor's consensus: Selective and nonselective nonsteroidal anti-inflammatory drugs and cardiovascular risk. *Am J Cardiol*. 106(6):873-84.

Frolov, A., Yang, L., Dong, H., Hammock, B. D., Crofford, L. J. (2013). Anti-inflammatory properties of prostaglandin E2: deletion of microsomal prostaglandin E synthase-1 exacerbates non-immune inflammatory arthritis in mice. *Prostaglandins Leukot Essent Fatty Acids*. 89(5):351-8.

Fryer, L. G., Parbu-Patel, A., Carling, D. (2002). The Anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. *J Biol Chem*. 277(28):25226-32.

Fuchs, H. J., Borowitz, D. S., Christiansen, D. H., Morris, E. M., Nash, M. L., Ramsey, B. W., Rosenstein, B. J., Smith, A. ., Wohl, M. E. (1994). Effect of Aerosolized Recombinant Human DNase on Exacerbations of Respiratory Symptoms and on Pulmonary Function in Patients with Cystic Fibrosis. *N Engl J Med.* 331:637-642.

Fujimoto, K., Kubo, K., Yamamoto, H., Yamaguchi, S., Matsuzawa, Y. (1999). Eosinophilic inflammation in the airway is related to glucocorticoid reversibility in patients with pulmonary emphysema. *Chest*. 115(3):697-702.

Fujino, T., Yamazaki, Y., Largaespada, D.A., Jenkins, N.A., Copeland, N.G., Hirokawa, K. & Nakamura, T. (2001) Inhibition of myeloid differentiation by Hoxa9, Hoxb8, and Meis homeoborcfenes. *Experimental Hematology*, 29, 856-863.

Funk, C. D. (2001). Prostaglandins and Leukotrienes: Advances in Eicosanoid Biology. *Science*. 294(5548):1871-1875.

Furze, R. C., Rankin, S. M. (2008). The role of the bone marrow in neutrophil clearance under homeostatic conditions in the mouse. *The FASEB Journal*. 22, 3111-3119.

Gaggar, A., Li, Y., Weathington, N., Winkler, M., Kong, M., Jackson, P., Blalock, J. E., Clancy, J. P. (2007). Matrix metalloprotease-9 dysregulation in lower airway secretions of cystic fibrosis patients. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 293(1), L96–L104.

Galdston, M., Melnick, E. L., Goldring, R. M., Levytska, V., Curasi, C. A., Davis, A. L. (1977). Interactions of neutrophil elastase, serum trypsin inhibitory activity, and smoking history as risk factors for chronic obstructive pulmonary disease in patients with MM, MZ, and ZZ phenotypes for alpha-1 antitrypsin. *Am Rev Respir Dis*. 116(5):837–846.

Gallagher, R., Collins, S., Trujillo, J., McCredie, K., Ahearn, M., Tsai, S., Metzgar, R., Aulakh, G., Ting, R., Ruscetti, F., Gallo, R. (1979). Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. *Blood*. 54(3):713–33.

Galvao, J., Davis, B., Tilley, M., Normando, E., Duchen, M. R., Cordeiro, M. F. (2014). Unexpected low-dose toxicity of the universal solvent DMSO. *FASEB J*. 28(3):1317-30.

Gao, J.L. & Murphy, P.M. (1993) Species and subtype variants of the n-formyl peptide chemotactic receptor reveal multiple important functional domains. *Journal of Biological Chemistry*. 268, 25395-25401.

Garavito, R. M., Malkowski, M. G., DeWitt, D. L. (2002). The structures of prostaglandin endoperoxide H synthases-1 and -2. *Prostaglandins & other lipid mediators*. 68-69:129-52.

Garcia-Faroldi, G., Melo, F. R., Bruemmer, D., Conneely, O. M., Pejler, G., Lundequist, A. (2014). Nuclear receptor 4a3 (nr4a3) regulates murine mast cell responses and granule content. *PLoS One*. 9(2):e89311.

Garnock-Jones, K. P. (2015). Roflumilast: A Review in COPD. *Drugs*.75(14):1645-56.

Gautam, S., Kirschnek, S., Wiesmeier, M., Vier, J., Häcker, G. (2013). Roscovitine-induced Apoptosis in Neutrophils and Neutrophil Progenitors Is Regulated by the Bcl-2-Family Members Bim, Puma, Noxa and Mcl-1. *PLoS One*. 8(11):e79352.

Gavett, S. H., Madison, S. L., Chulada, P. C., Scarborough, P. E., Qu, W., Boyle, J. E., Tiano, H. F., Lee, C. A., Langenbach, R., Roggli, V. L., Zeldin, D. C. (1999). Allergic lung responses are increased in prostaglandin H synthase-deficient mice. *J Clin Invest*. 104:721–732.

Geijtenbeek, T.B.H., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C.F., Adema, G.J., van Kooyk, Y. & Figdor, C.G. (2000) Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell*, 100, 575-585.

Gilroy, D. W., Colville-Nash, P. R., Willis, D., Chivers, J., Paul-Clark, M. J., Willoughby, D. A. (1999). Inducible cyclooxygenase may have anti-inflammatory properties. *Nat Med*. 5:698–701.

Gjertsen, B. T., Mellgren, G., Otten, A., Maronde, E., Genieser, H. G., Jastorff, B., Vintermyr, O. K., McKnight, G. S., Doskeland, S. O. (1995) Novel (Rp)-cAMPS analogs as tools for inhibition of cAMP-kinase in cell culture. Basal cAMP-kinase activity modulates interleukin-1 action. *J. Biol. Chem*. 270:20599–20607.

Gossett, K. A., MacWilliams, P. S. (1982). Ultrastructure of canine toxic neutrophils. *Am J Vet Res*. 43:1634-1637.

Gottlieb, D. J., Stone, P. J., Sparrow, D., Gale, M. E., Weiss, S. T., Snider, G. L., O'Connor, G. T. (1996). Urinary desmosine excretion in smokers with and without rapid decline of lung function: the Normative Aging Study. *Am J Respir Crit Care Med.* 154:1290–1295.

Graham, K. A., Buick, R. N. (1988). Sodium Butyrate Induces Differentiation in Breast Cancer Cell Lines Expressing the Estrogen Receptor. *J. Cell.Physiol*. 136:63.

Griffiths, G., Hollinshead, R., Hemmings, B. A., Nigg, E. A. (1990) Ultrastructural-localiyation of the regulatory (RII) subunit of cyclic AMP-dependent protein-kinase to subcellular compartments active in endocytosis and recycling of membrane-receptors. *Journal of Cell Science*. 96:691-703.

Gürsel, G., Türktas, H., Gökçora, N., Tekin, I. O. (1997). Comparison of sputum and serum eosinophil cationic protein (ECP) levels in nonatopic asthma and chronic obstructive pulmonary disease. *J Asthma*. 34(4):313-9.

Hafezi-Moghadam, A., Thomas, K. L., Prorock, A. J., Huo, Y., Ley, K. (2001). L-Selectin Shedding Regulates Leukocyte Recruitment. *The Journal of Experimental Medicine.* 193: 863-872.

Hahn-Windgassen, A., Nogueira, V., Chen, C.-C., Skeen, J. E., Sonenberg, N., Hay, N. (2005). Akt Activates the Mammalian Target of Rapamycin by Regulating Cellular ATP Level and AMPK Activity. *The Journal of Biological Chemistry.* 280:32081-32089.

Ham, E. A., Soderman, D. D., Zanetti, M. E., Dougherty, H. W., McCauley, E., Kuehl, F. A. Jr. (1983). Inhibition by prostaglandins of leukotriene B4 release from activated neutrophils. *PNAS*. 80(14):4349-53.

Hamberg, M., Samuelsson, B. (1971). On the Metabolism of Prostaglandins E, and E, in Man. *Journal of Biological Chemistry*. 246 (22): 6713-6721.

Hanna, R. N., Carlin, L. M., Hubbeling, H. G., Nackiewicz, D., Green, A. M., Punt, J. A., Geissmann, F., Hedrick, C. C. (2011). The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C-monocytes. *Nat Immunol*. 12(8):778-85.

Hanslick, J. L., Lau, K., Noguchi, K. K., Olney, J. W., Zorumski, C. F., Mennerick, S., Farber, N. B. (2009). Dimethyl Sulfoxide (DMSO) Produces Widespread Apoptosis in the Developing Central Nervous System. *Neurobiol Dis*. 34(1): 1–10.

Hardie, D. G. (2007). AMPK and Raptor: matching cell growth to energy supply. *Mol. Cell.* 30:263-265.

Hardie, D.G. (2008). Role of AMP-activated protein kinase in the metabolic syndrome and in heart disease. *FEBS Lett.* 582:81-89.

Hartl, D., Latzin, P., Hordijk, P., Marcos, V., Rudolph, C., Woischnik, M., Krauss-Etschmann, S., Koller, B., Reinhardt, D., Roscher, A. A., Roos, D., Griese, M. (2007). Cleavage of CXCR1 on neutrophils disables bacterial killing in cystic fibrosis lung disease. *Nature Medicine.* 13(12):1423–1430.

Haslett C. (1997). Granulocyte apoptosis and inflammatory disease. *Br Med Bull*. 53(3):669–83.

Haslett, C. (1999) Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *American Journal of Respiratory and Critical Care Medicine*, 160, S5-S11.

Hautamaki, R. D., Kobayashi, D. K., Senior, R. M., Shapiro, S. D. (1997). Macrophage elastase is required for cigarette smoke-induced emphysema in mice. Science 277:2002-2004.

Hawk, J. D., Abel, T. (2011). The role of NR4A transcription factors in memory formation. *Brain Res Bull*. 85(1-2): 21–29.

Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessi, D. R. and Hardie, D. G. (2003). Complexes between the LKB1 tumor suppressor, STRADa/b and MO25a/b are upstream kinases in the AMPK cascade. *J. Biol.* 2(28):1-16.

Hayashi, H., Fujii, Y., Watanabe, K., Urade, Y., Hayaishi, O. (1989). Enzymatic conversion of prostaglandin H2 to prostaglandin F2 alpha by aldehyde reductase from human liver: comparison to the prostaglandin F synthetase from bovine lung. *The Journal of biological chemistry*. 264(2):1036-40.

Helbling, J. C., Minni, A. M., Pallet, V., Moisan, M. P. (2014). Stress and glucocorticoid regulation of NR4A genes in mice. *J Neurosci Res*. 92(7):825-34.

Hill, A. T., Bayley, D., Stockley, R. A. (1999). The interrelationship of sputum inflammatory markers in patients with chronic bronchitis. *Am J Respir Crit Care Med*. 160(3):893-8.

Hill, A. T., Campbell, E. J., Hill, S. L., Bayley, D. L., Stockley, R. A. (2000). Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis. *Am J Med*. 109:288-295.

Hippisley-Cox, J., Coupland, C. (2005). Risk of myocardial infarction in patients taking cyclo-oxygenase-2 inhibitors or conventional non-steroidal anti-inflammatory drugs: population based nested case control analysis. *BMJ*. 330:1–7.

Hodge, S. J., Hodge, G. L., Holmes, M., Reynolds, P. N. (2004). Flow Cytometric Characterization of Cell Populations in Bronchoalveolar Lavage and Bronchial Brushings From Patients With Chronic Obstructive Pulmonary Disease. *Cytometry Part B (Clinical Cytometry)*. 61B:27–34.

Hoenderdos, K., Hirst, R. A., Porter, L., Chen, C., Lodge, K., O'Callaghan, C., Chilvers, E. R., Condliffe, A. M. (2013). COPD: mechanisms of host defence. S115 The effects of hypoxia on neutrophil-mediated tissue damage in the lung. *Thorax*. 68:A60.

Hoffman, R., Tong, J., Brandt, J., Traycoff, C., Bruno, E., McGuire, B. W., Gordon, M. S., McNiece, I., Srour, E. F. (1993). The *in vitro* and *In vivo* effects of stem cell factor on human hematopoiesis. *Stem Cells*. 2:76-82.

Hogg, J. C., Walker, B. A. (1995). Polymorphonuclear leucocyte traffic in lung inflammation. *Thorax*. 50:819-820.

Holla, V. R., Mann, J. R., Shi, Q., DuBois, R. N. (2006). Prostaglandin E2 regulates the nuclear receptor NR4A2 in colorectal cancer. *J Biol Chem*. 281(5):2676-82.

Hotta, K., Niwa, M., Hara, A., Ohno, T., Wang, X., Matsuno, H. *et al.* (2001). The loss of susceptibility to apoptosis in exudated tissue neutrophils is associated with their nuclear factor-kappa B activation. *European Journal of Pharmacology*. 433:17-27.

Hsu, H. C., Zhou, T., Mountz, J. D. (2004). Nur77 family of nuclear hormone receptors. *Curr Drug Targets Inflamm Allergy*. 3(4):413-23.

Hubbard, N. E., Lee, S., Lim, D., Erickson, K. L. (2001). Differential mRNA expression of prostaglandin receptor subtypes in macrophage activation. *Prostaglandins Leukot Essent Fatty Acids*. 65(5-6):287-94.

Hurst, S. M., Wilkinson, T. S., McLoughlin, R. M., Jones, S., Horiuchi, S., Yamamoto, N. *et al.* (2001). IL-6 and Its Soluble Receptor Orchestrate a Temporal Switch in the Pattern of Leukocyte Recruitment Seen during Acute Inflammation. *Immunity*. 14:705-714.

Huynh, M. L., Fadok, V. A., Henson, P. M. (2002). Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-β1 secretion and the resolution of inflammation. *J Clin Investig*. 109:41-50.

Idziorek, T., Estaquier, J., De Bels, F., Ameisen, J.-C. (1995). YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *J. Immun. Meth*. 185:249-258.

Ikeda-Matsuo, Y., Tanji, H., Narumiya, S., Sasaki, Y. (2011). Inhibition of prostaglandin E2 EP3 receptors improves stroke injury via anti-inflammatory and anti-apoptotic mechanisms. *J Neuroimmunol*. 238(1-2):34-43.

Ikegami, R., Sugimoto, Y., Segi, E., Katsuyama, M., Karahashi, H., Amano, F., Maruyama, T., Yamane, H., Tsuchiya, S., Ichikawa, A. (2001). The Expression of Prostaglandin E Receptors EP2 and EP4 and Their Different Regulation by Lipopolysaccharide in C3H/HeN Peritoneal Macrophages. *J Immunol.* 166(7):4689-96.

Inazu, N., Ruepp, B., Wirth, H., Wermuth, B. (1992). Carbonyl reductase from human testis: purification and comparison with carbonyl reductase from human brain and rat testis. *Biochimica et biophysica acta*. 1116(1):50-6.

Insel, P.A., Zhang, L., Murray, F., Yokouchi, H. & Zambon, A.C. (2012) Cyclic AMP is both a pro-apoptotic and anti-apoptotic second messenger. *Acta Physiol*., 204, 277–287.

Jakobsson, P. J., Thorén, S., Morgenstern, R., Samuelsson, B. (1999). Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc Natl Acad Sci U S A*. 96(13):7220-5.

Janeway, C. A., Travers, P., Walport M., & Shlomchik M.J. (2001). *Immunobiology: the immune system in health and disease.* 5th ed. New York: Garland Publishing.

Jankovic, J., Chen, S., Le, W. D. (2005). The role of Nurr1 in the development of dopaminergic neurons and Parkinson's disease. *Prog Neurobiol*. 77(1–2):128–138.

Janoff, A., Sloan, B., Weinbaum, G., Damiano, V., Sandhaus, R. A., Elias, J., Kimbel, P. (1977). Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled protease. *Am Rev Respir Dis*. 115(3):461-78.

Jen, R., Rennard, S. I., Sin, D. D. (2012). Effects of inhaled corticosteroids on airway inflammation in chronic obstructive pulmonary disease: a systematic review and meta-analysis. *Int. J. Chron. Obstr. Pulm. Dis*. 7:587–595.

Jiang, Y. J., Lu, B., Choy, P. C., Hatch, G. M. (2003). Regulation of cytosolic phospholipase A2, cyclooxygenase-1 and -2 expression by PMA, TNFα, LPS and M-CSF in human monocytes and macrophages. *Mol Cell Biochem*. 246:31-38.

Jiang, H., Weyrich, A. S., Zimmerman, G. A., McIntyre, T. M. (2004). Endothelial cell confluence regulates cyclooxygenase-2 and prostaglandin E2 production that modulate motility. *J Biol Chem*. 279(53):55905-13.

Jimenez, M. F., Watson, R. W., Parodo, J., Evans, D., Foster, D., Steinberg, M., Rotstein, O. D., Marshall, J. C. (1997). Dysregulated expression of neutrophil apoptosis in the systemic inflammatory response syndrome. *Arch. Surg*. 132:1263–1270.

Jimenez, J. L., Punzon, C., Navarro, J., Munoz-Fernandez, M. A., Fresno, M. (2001). Phosphodiesterase 4 inhibitors prevent cytokine secretion by T lymphocytes by inhibiting nuclear factor-kappaB and nuclear factor of activated T cells activation. *J Pharmacol Exp Ther*. 299:753-759.

Jinnai, N., Sakagami, T., Sekigawa, T., Kakihara, M., Nakajima, T., Yoshida, K., Goto, S., Hasegawa, T., Koshino, T., Hasegawa, Y., Inoue, H., Suzuki, N., Sano, Y., Inoue, I. (2004). Polymorphisms in the prostaglandin E2 receptor subtype 2 gene confer susceptibility to aspirin-intolerant asthma: a candidate gene approach. *Hum. Molec. Genet.* 13:3203-3217.

Jog, N. R., Rane, M. J., Lominadze, G., Luerman, G. C., Ward, R. A., McLeish, K. R. (2007). The actin cytoskeleton regulates exocytosis of all neutrophil granule subsets. *Am J Physiol Cell Physiol.* 292(5):C1690-700.

Jones, J., Morgan, B. P. (1995). Apoptosis is associated with reduced expression of complement regulatory molecules, adhesion molecules and other receptors on polymorphonuclear leucocytes: Functional relevance and role in inflammation. *Immunology.* 86:651–660.

Jozsef, L., Khreiss, T., Filep, J. G. (2004). CpG motifs in bacterial DNA delay apoptosis of neutrophil granulocytes. *FASEB J*. 18:1776–1778.

Juan, Z., Bian, H.-J., Li, X.-X., Liu, X.-B., Sun, J.-P., Na, L., Yun, Z. & Ji, X.-P. (2010) ERK-MAPK Signaling Opposes Rho-Kinase to Reduce Cardiomyocyte Apoptosis in Heart Ischemic Preconditioning. *Molecular Medicine*, 16, 307-315.

Jun, H. S., Lee, Y. M., Song, K. D., Mansfield, B. C., Chou, J. Y. (2011). G-CSF improves murine G6PC3-deficient neutrophil function by modulating apoptosis and energy homeostasis. *Blood*. 117(14):3881-92.

Juss, J. K., Hayhoe, R. P., Owen, C. E., Bruce, I., Walmsley, S. R., Cowburn, A. S., Kulkarni, S., Boyle, K. B., Stephens, L., Hawkins, P. T., Chilvers, E. R., Condliffe, A. M. (2012). Functional Redundancy of Class I Phosphoinositide 3-Kinase (PI3K) Isoforms in Signaling Growth Factor-Mediated Human Neutrophil Survival. *PLoS One*. 7(9):e45933.

Kampitsch, N., Kónya, V., Schuligoi, R., Heinemann, A. (2012). The role of PGE2 EP4 receptors in the regulation of endothelial barrier function. *BMC Pharmacol Toxicol*. 13(Suppl 1):A20.

Kanamori, Y., Niwa, M., Kohno, K., Al-Essa, L. Y., Matsuno, H., Kozawa, O., Uematsu, T. (1997). Migration of neutrophils from blood to tissue: alteration of modulatory effects of prostanoid on superoxide generation in rabbits and humans. *Life Sci*. 60(16):1407-17.

Kanefsky, J., Lenburg, M., Hai, C. M. (2006). Cholinergic receptor and cyclic stretch-mediated inflammatory gene expression in intact ASM. *Am J Respir Cell Mol Biol*. 34(4):417-25.

Kasahara, Y., Tuder, R. M., Taraseviciene-Stewart, L., Le Cras, T. D., Abman. S., Hirth, P. K., Waltenberger, J., Voelkel, N. F. (2000). Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Investig.* 106:1311-1319.

Kato, T., Kutsuna, H., Oshitani, N., Kitagawa, S. (2006). Cyclic AMP delays neutrophil apoptosis via stabilization of Mcl-1. *FEBS Lett*. 580(19):4582-6.

Katsuyama, M., Ikegami, R., Karahashi, H., Amano, F., Sugimoto, Y., Ichikawa, A. (1998). Characterization of the LPS-stimulated expression of EP2 and EP4 prostaglandin E receptors in mouse macrophage-like cell line, J774.1. Biochem Biophys Res Commun. 251(3):727-31.

Ke, N., Claassen, G., Yu, D. H., Albers, A., Fan, W., Tan, P., Grifman, M., Hu, X., Defife, K., Nguy, V., Meyhack, B., Brachat, A., Wong-Staal, F., Li, Q. X. (2004). Nuclear hormone receptor NR4A2 is involved in cell transformation and apoptosis. *Cancer Res*. 64(22):8208-12.

Keatings, V. M., Barnes, P. J. (1997). Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma and normal subjects. *Am J Respir Crit Care Med*. 155: 449-453.

Keatings, V. M., Collins, P. D., Scott, D. M., Barnes, P. J. (1996). Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med*. 153(2):530-4.

Keatings, V. M., Jatakanon, A., Worsdell, Y. M., Barnes, P. J. (1997). Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. *Am J Respir Crit Care Med*. 155(2):542-8.

Kennedy, A. D., DeLeo, F. R. (2009). Neutrophil apoptosis and the resolution of infection. *Immunol Res*. 43(1-3):25-61.

Kennedy, P. A., Kilpatrick, L. E. (2013). Neutrophil Inflammation in COPD. In: Rogers, T. J., Criner, G. J., Cornwell, W. D. *Smoking and Lung Inflammation: Basic, Pre-Clinical and Clinical Research.* New York: Springer, 68.

Kerr, J. F., Wyllie, A. H., Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 26(4):239-57.

Kickler, K., Maltby, K., Ni Choileain, S., Stephen, J., Wright, S., Hafler, D. A., Jabbour, H. N., Astier, A. L. (2012). Prostaglandin E2 affects T cell responses through modulation of CD46 expression. *J Immunol*. 188(11):5303-10.

Kilpatrick L. E., Sun, S., Mackie, D., Baik, F., Li, H., Korchak, H. M. (2006). Regulation of TNF mediated antiapoptotic signaling in human neutrophils: Role of d-PKC and ERK1/2. *J Leukoc Biol.* 80:1512–1521.

Kilpatrick, L. E., Lee, J. Y., Haines, K. M., Donald E. Campbell, Sullivan, K. E., Korchak, H. M. (2002). A role for PKC-d and PI 3-kinase in TNFa-mediated antiapoptotic signaling in the human neutrophil. *Am J Physiol Cell Physiol.* 283:C48–C57.

Kim, J. H., Auerbach, J. M., Rodríguez-Gómez, J. A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S. H., Nguyen, J., Sánchez-Pernaute, R., Bankiewicz, K., McKay, R. (2002). Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature*. 418(6893):50–56.

King, P. T. (2015). Inflammation in chronic obstructive pulmonary disease and its role in cardiovascular disease and lung cancer. *Clin Transl Med*. 4: 26.

Kirschnek, S., Vier, J., Gautam, S., Frankenberg, T., Rangelova, S., Eitz-Ferrer, P., Grespi, F., Ottina, E., Villunger, A., Haecker, H. & Haecker, G. (2011) Molecular analysis of neutrophil spontaneous apoptosis reveals a strong role for the pro-apoptotic BH3-only protein Noxa. *Cell Death and Differentiation*, 18, 1805-1814.

Klausen, P., Bjerregaard, M. D., Borregaard, N., Cowland, J. B. (2004). End-stage differentiation of neutrophil granulocytes *In vivo* is accompanied by up-regulation of p27kip1 and down-regulation of CDK2, CDK4, and CDK6. *Journal of Leukocyte Biology*. 75(3):569-578.

Kliber, A., Lynd, L. D., Sin, D. D. (2010). The effects of long-acting bronchodilators on total mortality in patients with stable chronic obstructive pulmonary disease. *Respir Res*. 11:56.

Knoepfler, P.S., Sykes, D.B., Pasillas, M. & Kamps, M.P. (2001) HoxB8 requires its Pbx-interaction motif to block differentiation of primary myeloid progenitors and of most cell line models of myeloid differentiation. *Oncogene*, 20, 5440-5448.

Kobayashi, S. D., Voyich, J. M., Buhl, C. L., Stahl, R. M., DeLeo, F. R. (2002). Global changes in gene expression by human polymorphonuclear leukocytes during receptor-mediated phagocytosis: cell fate is regulated at the level of gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 99:6901–6906.

Koethe, S. M., Kuhnmuench, J. R., Becker, C. G. (2000). Neutrophil Priming by Cigarette Smoke Condensate and a Tobacco Anti-Idiotypic Antibody. *Am J Pathol*. 157(5): 1735–1743.

Konger, R. L., Billings, S. D., Thompson, A. B., Morimiya, A., Ladenson, J. H., Landt, Y., Pentland, A. P., Badve, S. (2005). Immunolocalization of low-affinity prostaglandin E receptors, EP and EP, in adult human epidermis. *J Invest Dermatol*. 124(5):965-70.

Kopperud, R., Krakstad, C., Selheim, F., Døskeland, S. O. (2003). cAMP effector mechanisms. Novel twists for an 'old' signaling system. *FEBS Lett*. 546(1):121-6.

Korsgren, M., Linden, M., Entwistle, N., Cook, J., Wollmer, P., Andersson, M., Larsson, B., Greiff, L. (2012). Inhalation of LPS induces inflammatory airway responses mimicking characteristics of chronic obstructive pulmonary disease. *Clin Physiol Funct Imaging*. 32(1):71-9.

Kovalovsky, D., Refojo, D., Liberman, A. C., Hochbaum, D., Pereda, M. P., Coso, O. A., Stalla, G. K., Holsboer, F., Arzt, E. (2002). Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. *Mol Endocrinol*. 16:1638–1651.

Kovarova, M., Koller, B. H. (2014). PGE_2 promotes apoptosis induced by cytokine deprivation through EP3 receptor and induces Bim in mouse mast cells. *PLoS One*. 9(7):e102948.

Kozu, Y., Gon, Y., Maruoka, S., Kazumichi, K., Sekiyama, A., Kishi, H., Nomura, Y., Ikeda, M., Hashimoto, S. (2015). Protocadherin-1 is a glucocorticoid-responsive critical regulator of airway epithelial barrier function. *BMC Pulm Med*. 15:80.

Krakstad, C., Christensen, A. E., Døskeland, S. O. (2004). cAMP protects neutrophils against TNF--induced apoptosis by activation of cAMP-dependent protein kinase, independently of exchange protein directly activated by cAMP (Epac). *Journal of Leukocyte*. 76(3):641-7.

Kunisch E, Jansen, A., Kojima, F., Löffler, I., Kapoor, M., Kawai, S., Rubio, I., Crofford, L. J., Kinne, R. W. (2009). Prostaglandin E2 differentially modulates proinflammatory/prodestructive effects of TNF-alpha on synovial fibroblasts via specific E prostanoid receptors/cAMP. *J Immunol*. 183(2):1328-36.

Kurai, M., Robbins, R. A., Koyama, S., Amano, J., Hayden, J. M. (2012). Acetylcholine stimulation of human neutrophil chemotactic activity is directly inhibited by tiotropium involving Gq and ERK-1/2 regulation. *Southwest J Pulm Crit Care.* 5:152-68.

Kwok, R. P. S., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G. E., Green, M. R., Goodman, R. H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature*. 370:223–226.

Lacoste, J. Y., Bousquet, J., Chanez, P., Van Vyve, T., Simony-Lafontaine, J., Lequeu, N., Vic, P., Enander, I., Godard, P., Michel, F. B. (1993). Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis, and chronic obstructive pulmonary disease. *J Allergy Clin Immunol*. 92(4):537–548.

Laflamme, C., Filion, C., Bridge, J. A., Ladanyi, M., Goldring, M. B., Labelle, Y. (2003). The homeotic protein Six3 is a coactivator of the nuclear receptor NOR-1 and a corepressor of the fusion protein EWS/NOR-1 in human extraskeletal myxoid chondrosarcomas. *Cancer Res*. 63(2):449-54.

Laing, K. J., Secombes, C. J. (2004). Chemokines. *Developmental & Comparative Immunology.* 28:443-460. Review.

Lamkanfi, M., Declercq, W., Kalai, M., Saelens, X. & Vandenabeele, P. (2002) Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death and Differentiation*, 9, 358-361.

Lammi, J., Aarnisalo, P. (2008). FGF-8 stimulates the expression of NR4A orphan nuclear receptors in osteoblasts. *Mol Cell Endocrinol*. 295(1-2):87-93.

Langenbach, R., Loftin, C., Lee, C., Tiano, H. (1999). Cyclooxygenase knockout mice-Models for elucidating isoform-specific functions. *Biochem Pharmacol*. 58:1237–1246.

Langereis, J., Schweizer, R., Lammers, J. W., Koenderman, L., Ulfman, L. (2011). A unique protein profile of peripheral neutrophils from COPD patients does not reflect cytokine-induced protein profiles of neutrophils *in vitro*. *BMC Pulm Med*. 11:44.

Laurell, C.-B., Eriksson, S. (1963). The electrophoretic alpha-1-globulin pattern of serum in alpha-1-antitrypsin deficiency. *Scand J Clin Lab Invest*. 15:132–140.

Lawrence, H.J., Helgason, C.D., Sauvageau, G., Fong, S., Izon, D.J., Humphries, R.K. & Largman, C. (1997) Mice bearing a targeted interruption of the homeoborcfene HOXA9 have defects in myeloid, erythroid, and lymphoid hematopoiesis. *Blood*. 89:1922-1930.

Le, W. D., Xu, P., Jankovic, J., Jiang, H., Appel, S. H., Smith, R. G., Vassilatis, D. K. (2003). Mutations in NR4A2 associated with familial Parkinson disease. *Nat Genet.* 33(1):85-9.

Lee, M. K., Nikodem, V. M. (2004). Differential role of ERK in cAMP-induced Nurr1 expression in N2A and C6 cells. *Neuroreport*. 15:99–102.

Lee, W. L., Grinstein, S. (2004). The Tangled Webs That Neutrophils Weave. *Immunology.* 303 (5663): 1477-1478.

Le Poul E, Loison C, Struyf S, Springael, J.Y., Lannoy, V., Decobecq, M. E., Brezillon, S., Dupriez, V., Vassart, G., Van Damme, J., Parmentier, M., Detheux, M. (2003). Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *J Biol Chem.* 278:25481–25489.

Li, Q. X., Ke, N., Sundaram, R., Wong-Staal, F. (2006). NR4A1, 2, 3--an orphan nuclear hormone receptor family involved in cell apoptosis and carcinogenesis. *Histol Histopathol*. 21(5):533-40.

Liles, W. C., Dale, D. C., Klebanoff, S. J. (1995). Glucocorticoids Inhibit Apoptosis of Human Neutrophils. *Blood*. 86(8):3181-3188.

Lim, H., Dey, S. K. (2000). PPAR delta functions as a prostacyclin receptor in blastocyst implantation. *Trends in endocrinology and metabolism: TEM*. 11(4):137-42.

Lim, S., Roche, N., Oliver, B. G., Mattos, W., Barnes, P. J., Fan, C. K. (2000). Balance of matrix metalloprotease-9 and tissue inhibitor of metalloprotease-1 from alveolar macrophages in cigarette smokers. Regulation by interleukin-10. *Am J Respir Crit Care Med*. 162:1355-1360.

Liu, Z.-G., Smith, S. W., McLaughlin, K. A., Schwartz, L. M., Osborne, B. A. (1994). Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. *Nature*. 367:281-284.

Liu, H., Lazarus, S. C., Caughey, G. H., Fahy, J. V. (1999). Neutrophil elastase and elastase-rich cystic fibrosis sputum degranulate human eosinophils *in vitro*. *Am J Physiol*. 276(1 Pt 1):L28-34.

Liu, J. & Lin, A.N. (2005) Role of JNK activation in apoptosis: A double-edged sword. *Cell Research*. 15:36-42.

Liu, K. G., Lambert, M. H., Ayscue, A. H., Henke, B. R., Leesnitzer, L. M., Oliver, W. R., Plunket, K. D., Xu, H. E., Sternbach, D. D., Willson, T. M. (2001). Synthesis and biological activity of L-tyrosine-based PPARgamma agonists with reduced molecular weight. *Bioorg. Med. Chem. Lett.* 11:3111-3113.

Lorne, E., Zhao, X., Zmijewski, J. W., Liu, G., Park, Y. J., Tsuruta, Y., Abraham, E. (2009). Participation of mammalian target of rapamycin complex 1 in Toll-like receptor 2- and 4-induced neutrophil activation and acute lung injury. *Am J Respir Cell Mol Biol.* 41(2):237-45.

Lotz, S., Aga, E., Wilde, I., van Zandbergen, G., Hartung, T., Solbach, W., Laskay, T. (2004). Highly purified lipoteichoic acid activates neutrophil granulocytes and delays their spontaneous apoptosis via CD14 and TLR2. *J. Leukocyte Biol*. 75:467–477.

Lu, Z., Zhang, C., Zhai, Z. (2005). Nucleoplasmin regulates chromatin condensation during apoptosis. *Proc Natl Acad Sci U S A*. 102(8):2778-83.

Lu, D., Han, C., Wu, T. (2013). 15-Hydroxyprostaglandin Dehydrogenase-derived 15-Keto-prostaglandin E2 Inhibits Cholangiocarcinoma Cell Growth through Interaction with Peroxisome Proliferator-activated Receptor-γ, SMAD2/3, and TAP63 Proteins. *J Biol Chem*. 288(27): 19484–19502.

Lundbäck, B., Lindberg, A., Lindstrom, M., Rönmark, E., Jonsson, A. C., Jönsson, E., Larsson, L. G., Andersson, S., Sandström, T., Larsson, K.; Obstructive Lung Disease in Northern Sweden Studies. (2003). Not 15 but 50% of smokers develop COPD? Report from the Obstructive Lung Disease in Northern Sweden Studies. *Respir Med*. 97: 115–122.

Lundborg, M., Dahlén, S. E., Johard, U., Gerde, P., Jarstrand, C., Camner, P., Låstbom, L. (2006). Aggregates of ultrafine particles impair phagocytosis of microorganisms by human alveolar macrophages. *Environ Res*. 100(2):197-204.

Lundborg, M., Johard, U., Låstbom, L., Gerde, P., Camner, P. (2001). Human alveolar macrophage phagocytic function is impaired by aggregates of ultrafine carbon particles. *Environ Res*. 86(3):244-53.

Luo, H. R., Loison, F. (2008). Constitutive neutrophil apoptosis: Mechanisms and regulation. *American Journal of Hematology*. 83: 288-295. Review.

MacKenzie, S. J., Baillie, G. S., McPhee, I., MacKenzie, C., Seamons, R., McSorley, T., Millen, J., Beard, M. B., van Heeke, G., Houslay, M. D. (2002). Long PDE4 cAMP specific phosphodiesterases are activated by protein kinase A-mediated phosphorylation of a single serine residue in Upstream Conserved Region 1 (UCR1). *Br J Pharmacol*. 136(3):421-33.

MacNee, W. (2001). Oxidative stress and lung inflammation in airways disease. *Eur J Pharmacol*. 429:195-207.

MacNee, W. (2006). ABC of chronic obstructive pulmonary disease Pathology, pathogenesis, and pathophysiology. *BMJ*. 332(7551): 1202–1204.

MacNee, W., Wiggs, B., Belzberg, A. S., Hogg, J. C. (1989). The effect of cigarette smoking on neutrophil kinetics in human lungs. *N Engl J Med*. 321(14):924-8.

Majo, J., Ghezzo, H., Cosio, M. G. (2001). Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. *Eur Respir J*. 17:946-953.

Makam, M., Diaz, D., Laval, J., Gernez, Y., Conrad, C. K., Dunn, C. E., Davies, Z. A., Moss, R. B., Herzenberg, L. A., Herzenberg, L. A., Tirouvanziam, R. (2009). Activation of critical, host-induced, metabolic and stress pathways marks neutrophil entry into cystic fibrosis lungs. *Proc Natl Acad Sci U S A.* 106(14):5779-83.

Makris, D., Vrekoussis, T., Izoldi, M., Alexandra, K., Katerina, D., Dimitris, T., Michalis, A., Tzortzaki, E., Siafakas, N. M., Tzanakis, N. (2009). Increased apoptosis of neutrophils in induced sputum of COPD patients. *Respir Med*. 103(8):1130-5.

Mannino, D. M., Homa, D. M., Akimbani, I. J. (2002). COPD surveillance – United States. *MMWR*. 51: 3–16.

Mardini, I. A., FitzGerald, G. A. (2001). Selective inhibitors of cyclooxygenase-2: a growing class of anti-inflammatory drugs. *Mol Interv*. 1:30–38.

Mark B. Hampton, M. B., Vissers, M. C. M., Keenan, J. I., Winterbourn, C. C. (2002). Oxidant-mediated phosphatidylserine exposure and macrophage uptake of activated neutrophils: possible impairment in chronic granulomatous disease. *Journal of Leukocyte Biology*. 71(5): 775-781.

Marley, S.B., Hadley, C.L. & Wakelin, D. (1994) Effect of genetic variation on induced neutrophilia in mice. *Infection and Immunity*. 62:4304-4309.

Marnett, L. J., Rowlinson, S. W., Goodwin, D. C., Kalgutkar, A. S., Lanzo, C. A. (1999). Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. *The Journal of biological chemistry*. 274(33):22903-6.

Martí-Lliteras, P., Regueiro, V., Morey, P., Hood, D. W., Saus, C., Sauleda, J., Agustí, A. G., Bengoechea, J. A., Garmendia, J. (2009). Nontypeable Haemophilus influenzae clearance by alveolar macrophages is impaired by exposure to cigarette smoke. *Infect Immun*. 77(10):4232-42.

Martin, M. C., Dransfield, I., Haslett, C., Rossi, A. G. (2001). Cyclic AMP regulation of neutrophil apoptosis occurs via a novel protein kinase A-independent signaling pathway. *J Biol Chem*. 276(48):45041-50.

Martorell, L., Gentile, M., Rius, J., Rodríguez, C., Crespo, J., Badimon, L., Martínez-González, J. (2009). The hypoxia-inducible factor 1/NOR-1 axis regulates the survival response of endothelial cells to hypoxia. *Mol Cell Biol*. 29(21):5828-42.

Marwick, J. A., Dorward, D. A., Lucas, C. D., Jones, K. O., Sheldrake, T. A., Fox, S., Ward, C., Murray, J., Brittan, M., Hirani, N., Duffin, R., Dransfield, I., Haslett, C., Rossi, A. G. (2013). Oxygen levels determine the ability of glucocorticoids to influence neutrophil survival in inflammatory environments. *J Leukoc Biol*. 94(6):1285-92.

Mathias, J. R., Perrin, B. J., Liu, T. X., Kanki, J., Look, A. T., & Huttenlocher, A. (2006). Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. *Journal of Leukocyte Biology*. 80:1281-1288.

Matsuba, K. T., Van Eeden, S. F., Bicknell, S. G., Walker, B. A., Hayashi, S., Hogg, J. C. (1997). Apoptosis in circulating PMN: increased susceptibility in L-selectin-deficient PMN. *Am J Physiol*. 272(6-2):H2852-8.

Matzinger, P. (2002). The Danger Model: A Renewed Sense of Self. *Science*. 296 (5566): 301-305.

Mayr, M., Xu, Q. (2001). Smooth muscle cell apoptosis in arteriosclerosis. *Exp Gerontol*. 36(7):969-87.

McCoy, J. M., Walkenhorst, D. E., McCauley, K. S., Elaasar, H., Everett, J. R., Mix, K. S. (2015). Orphan nuclear receptor NR4A2 induces transcription of the immunomodulatory peptide hormone prolactin. *Journal of Inflammation*. 12:13.

McDonald, J. U., Cortini, A., Rosas, M., Fossati-Jimack, L., Ling, G.S., Lewis, K.J., Dewitt, S., Liddiard, K., Brown, G.D., Jones, S.A., Hallett, M.B., Botto, M. & Taylor, P.R. (2011) *In vivo* functional analysis and genetic modification of *in vitro*-derived mouse neutrophils. *Faseb Journal*. 25:1972-1982.

McDonald, P. P., Fadok, V. A., Bratton, D., Henson, P. M. (1999). Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF-ß in macrophages that have ingested apoptotic cells. *Journal of Immunology*. 163:6164-6172.

McEvoy, A. N., Murphy, E. A., Ponnio, T., Conneely, O. M., Bresnihan, B., FitzGerald, O., Murphy, E. P. (2002). Activation of Nuclear Orphan Receptor NURR1 Transcription by NF-κB and Cyclic Adenosine 5′-Monophosphate Response Element-Binding Protein in Rheumatoid Arthritis Synovial Tissue. *The Journal of Immunology*. 168(6): 2979-2987.

McGettrick, H. M., Hunter, K., Moss, P. A., Buckley, C. D., Rainger, G. E., Nash, G. B. (2009). Direct observations of the kinetics of migrating T cells suggest active retention by endothelial cells with continual bidirectional migration. *Journal of Leukocyte Biology*. 85:98-107.

McGrath, E. E., Marriott, H. M., Lawrie, A., Francis, S. E., Sabroe, I., Renshaw, S. A., Dockrell, D. H., Whyte, M. K. (2011). TNF-related apoptosis-inducing ligand (TRAIL) regulates inflammatory neutrophil apoptosis and enhances resolution of inflammation. *J Leukoc Biol*. 90(5):855-65.

McPhillips, K., Janssen, W. J., Ghosh, M., Byrne, A., Gardai, S., Remigio, L., Bratton, D. L., Kang, J. L., Henson, P. (2007). TNF-alpha inhibits macrophage clearance of apoptotic cells via cytosolic phospholipase A2 and oxidant-dependent mechanisms. *J Immunol*. 178(12):8117-26.

McStay, G.P., Salvesen, G.S. & Green, D.R. (2008) Overlapping cleavage motif selectivity of caspases: implications for analysis of apoptotic pathways. *Cell Death and Differentiation*, 15, 322-331.

Meagher, L. C., Cousin, J. M., Seckl, J. R., Haslett, C. (1996). Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *The Journal of Immunology*. 156(11):4422-4428.

Mehats, C., Jin, S. L., Wahlstrom, J., Law, E., Umetsu, D. T., Conti, M. (2003). PDE4D plays a critical role in the control of airway smooth muscle contraction. *FASEB J*. 17:1831-1841.

Meijer, M., Rijkers, G. T., van Overveld, F. J. (2013). Neutrophils and Emerging Targets for Treatment in Chronic Obstructive Pulmonary Disease. *Expert Rev Clin Immunol*. 9(11):1055-1068.

Meja, K. K., Barnes, P. J., Giembycz, M. A. (1997). Characterization of the prostanoid receptor(s) on human blood monocytes at which prostaglandin E2 inhibits lipopolysaccharride-induced tumour necrosis factor-α. *Br J Pharmacol*. 122:149-157.

Meshi, B., Vitalis, T. Z., Ionescu, D., Elliott, W. M., Liu, C., Wang, X. D., Hayashi, S., Hogg, J. C. (2002). Emphysematous lung destruction by cigarette smoke. The effects of latent adenoviral infection on the lung inflammatory response. *Am J Respir Cell Mol Biol*. 26: 52-57.

Mestas, J. & Hughes, C.C.W. (2004) Of mice and not men: Differences between mouse and human immunology. *Journal of Immunology*, 172, 2731-2738.

Metcalf, D. (2008) Hematopoietic cytokines. *Blood*, 111, 485-491.

Middelhoven PJ, Ager A, Roos D, Verhoeven AJ. (1997). Involvement of a metalloprotease in the shedding of human neutrophil Fc gammaRIIIB. *FEBS Lett.* 414(1):14-8.

Mix, K. S., McMahon, K., McMorrow, J. P., Walkenhorst, D. E., Smyth, A. M., Petrella, B. L., Gogarty, M., Fearon, U., Veale, D., Attur, M. G., Abramson, S. B., Murphy, E. P. (2012). Orphan nuclear receptor NR4A2 induces synoviocyte proliferation, invasion, and matrix metalloproteinase 13 transcription. *Arthritis Rheum*. 64(7):2126-36.

Miyaura, C., Inada, M., Suzawa, T., Sugimoto, Y., Ushikubi, F., Ichikawa, A., Narumiya, S., Suda, T. (2000). Impaired bone resorption to prostaglandin E2 in prostaglandin E receptor EP4-knockout mice. *J Biol Chem*. 275(26):19819-23.

Mizuno, R., Kamioka, Y., Kabashima, K., Imajo, M., Sumiyama, K., Nakasho, E., Ito, T., Hamazaki, Y., Okuchi, Y., Sakai, Y., Kiyokawa, E., Matsuda, M. (2014). *In vivo* imaging reveals PKA regulation of ERK activity during neutrophil recruitment to inflamed intestines. *J Exp Med*. 211(6):1123-36.

Moermans, C., Heinen, V., Nguyen, M., Henket, M., Sele, J., Manise, M., Corhay, J. L., Louis, R. (2011). Local and systemic cellular inflammation and cytokine release in chronic obstructive pulmonary disease. *Cytokine*. 56(2):298-304.

Mohan, H. M., Aherne, C. M., Rogers, A. C., Baird, A. W., Winter, D. C., Murphy, E. P. (2012). Molecular pathways: the role of NR4A orphan nuclear receptors in cancer. *Clin Cancer Res*. 18(12):3223-8.

Moir, H., Hughes, M. G., Potter, S., Sims, C., Butcher, L. R., Davies, N. A., Verheggen, K., Jones, K. P., Thomas, A. W., Webb, R. (2010). Exercise-induced immunosuppression: roles of reactive oxygen species and 5=-AMP-activated protein kinase dephosphorylation within immune cells. *J Appl Physiol.* 108:1284–1292.

Moissoglu, K., Schwartz, M. A. (2006). Integrin signalling in directed cell migration. *Biology of the Cell.* 98:547-555.

Montminy, M.R., Gonzalez, G.A. & Yamamoto, K.K. (1990) Regulation of cAMP-inducible genes by CREB. *Trends in Neurosciences*, 13, 184-188.

Montuschi, P., Kharitonov, S. A., Ciabattoni, G., Barnes, P. J. (2003). Exhaled leukotrienes and prostaglandins in COPD. *Thorax*. 58:585-588.

Montuschi, P., Macagno, F., Valente, S., Fuso, L. (2013). Inhaled muscarinic acetylcholine receptor antagonists for treatment of COPD. *Curr Med Chem.* 20(12):1464-76.

Morozumi, T., Kubota, T., Sugita, N., Itagaki, M., Yoshie, H. (2004). Alterations of gene expression in human neutrophils induced by smoking cessation. *J Clin Periodontol*. 31:1110–1116.

Moulding, D. A., Akgul, C., Derouet, M., White, M. R. H., Edwards, S. W. (2001). BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis. *Journal of Leukocyte Biology.* 70(5):783-92.

Moulton, B. C., Fryer, A. D. (2011). Muscarinic receptor antagonists, from folklore to pharmacology; finding drugs that actually work in asthma and COPD. *Br J Pharmacol.* 163(1):44-52.

Mukherjee, D., Nissen, S. E., Topol, E. J. (2001). Risk of cardiovascular events associated with selective COX-2 inhibitors. *JAMA.* 286(8):954-9.

Mullican, S. E., Zhang, S., Konopleva, M., Ruvolo, V., Andreeff, M., Milbrandt, J., Conneely, O. M. (2007). Abrogation of nuclear receptors Nr4a3 and Nr4a1 leads to development of acute myeloid leukemia. *Nature Medicine.* 13:730 – 735.

Murakami, M., Nakashima, K., Kamei, D., Masuda, S., Ishikawa, Y., Ishii, T., Ohmiya, Y., Watanabe, K., Kudo, I. (2003). Cellular prostaglandin E2 production by membrane-bound prostaglandin E synthase-2 via both cyclooxygenases-1 and -2. *The Journal of biological chemistry*. 278(39):37937-47.

Murakami, M., Naraba, H., Tanioka, T., Semmyo, N., Nakatani, Y., Kojima, F., Ikeda, T., Fueki, M., Ueno, A., Oh, S., Kudo, I. (2000). Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. *The Journal of biological chemistry*. 275(42):32783-92.

Murdoch, C., Finn, A. (2000). Chemokine receptors and their role in inflammation and infectious diseases. *Blood.* 95, 3032-3043.

Murphy, B. M., O'Neill, A. J., Adrain, C., Watson, R. W., Martin, S. J. (2003). The apoptosome pathway to caspase activation in primary human neutrophils exhibits dramatically reduced requirements for cytochrome C. *J Exp Med.* 197(5):625-32.

Murphy, Caraher, M. (2015). Mcl-1 is vital for neutrophil survival. *Immunol. Res*. 62 (2):225-33.

Murray, P. J. (2005). The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription. *Proc Natl Acad Sci U S A.* 102 (24): 8686-91.

Nadel, J. A. (1991). Role of mast cell and neutrophil proteases in airway secretion. *Am Rev Respir Dis*. 144(3 Pt 2):S48-51.

Nakanishi, M., Kakumoto, M., Matsuura, K., Deyashiki, Y., Tanaka, N., Nonaka, T., Mitsui, Y., Hara, A. (1996). Involvement of two basic residues (Lys-17 and Arg-39) of mouse lung carbonyl reductase in NADP(H)-binding and fatty acid activation: site-directed mutagenesis and kinetic analyses. *Journal of biochemistry*. 120(2):257-63.

Nakayama, T., Yashiro, K., Inoue, Y., Matsuura, K., Ichikawa, H., Hara, A., Sawada, H. (1986). Characterization of pulmonary carbonyl reductase of mouse and guinea pig. *Biochimica et biophysica acta*. 882(2):220-7.

Napolitani, G., Acosta-Rodriguez, E. V., Lanzavecchia, A., Sallusto, F. (2009). Prostaglandin E2 enhances Th17 responses via modulation of IL-17 and IFN-gamma production by memory CD4+ T cells. *Eur J Immunol*. 39(5):1301–1312.

Neofytou, E., Tzortzaki, E. G., Chatziantoniou, A., Siafakas, N. M. (2012). DNA Damage Due to Oxidative Stress in Chronic Obstructive Pulmonary Disease (COPD). *Int J Mol Sci*. 13(12): 16853–16864.

Newton, R., Kuitert, L. M., Bergmann, M., Adcock, I. M., Barnes, P. J. (1997). Evidence for involvement of NF-κB in the transcriptional control of COX-2 gene expression by IL-1β. *Biochem Biophys Res Commun*. 237:28-32.

Ni, J., Watson, J. V., Cox, H., Karpas, A. (1993). Multiparameter flow cytometric analysis of a novel cytotoxin (factor 2) induced tumor cell membrane permeability. *Cytometry*. 14:281-286.

Ni, I., Ji, C., Vij, N. (2015). Second-hand cigarette smoke impairs bacterial phagocytosis in macrophages by modulating CFTR dependent lipid-rafts. *PLoS One*. 10(3):e0121200.

Nichol, G., Nix, A., Barnes, P. J., Chung, K. F. (1990). Prostaglandin F2α enhancement of capsaicin induced cough in man: modulation by β2-adrenergic and anticholinergic drugs. *Thorax*. 45:694-698.

Nick, J. A., Avdi, N. J., Young, S. K., McDonald, P. P., Billstrom, M. A., Henson, P. M., Johnson, G. L., Worthen, G. S. (1999). The p38 MAP Kinase Cascade and its Functional Significance. *Chest.* 116(1): 54S-55S.

Nigg, E.A., Schafer, G., Hilz, H. & Eppenberger, H.M. (1985) Cyclic-AMP-dependent protein-kinase type-II is associated with the golgi-complex and with centrosomes. *Cell.* 41:1039-1051.

Nishigaki, N., Negishi, M., Ichikawa, A. (1996). Two Gs-coupled prostaglandin E receptor subtypes, EP2 and EP4, differ in desensitization and sensitivity to the metabolic inactivation of the agonist. *Mol Pharmacol*. 50(4):1031-7.

Noguera, A., Sala, E., Pons, A. R., Iglesias, J., MacNee, W., Agustí, A. G. N. (2004). Expression of Adhesion Molecules During Apoptosis of Circulating Neutrophils in COPD. *Chest*. 125(5):1837-1842.

Nomiyama, T., Nakamachi, T., Gizard, F., Heywood, E. B., Jones, K. L., Ohkura, N., Kawamori, R., Conneely, O. M., Bruemmer, D. (2006). The NR4A orphan nuclear receptor NOR1 is induced by platelet-derived growth factor and mediates vascular smooth muscle cell proliferation. *J Biol Chem*. 281(44):33467-76.

Nomura, T., Lu, R., Pucci, M. L., Schuster, V. L. (2004). The Two-Step Model of Prostaglandin Signal Termination: *In vitro* Reconstitution with the Prostaglandin Transporter and Prostaglandin 15 Dehydrogenase. *Molecular Pharmacology*. 65(4):973-978.

O'Donnell, R., Breen, D., Wilson, S., Djukanovic, R. (2006). Inflammatory cells in the airways of COPD. *Thorax*. 61(5):448-454.

Oger, S., Mehats, C., Dallot, E., Cabrol, D., Leroy, M. J. (2005). Evidence for a role of phosphodiesterase 4 in lipopolysaccharide-stimulated prostaglandin E2 production and matrix metalloproteinase-9 activity in human amniochorionic membranes. *J Immunol*. 174:8082-8089.

Ohkura, N., Hijikuro, M., Yamamoto, A., Miki, K. (1994). Molecular cloning of a novel thyroid/steroid receptor superfamily gene from cultured rat neuronal cells. *Biochem Biophys Res Commun*. 205(3):1959-65.

Ohkubo, T., Ohkura, N., Maruyama, K., Sasaki, K., Nagasaki, K., Hanzawa, H., Tsukada, T., Yamaguchi, K. (2000). Early induction of the orphan nuclear receptor NOR-1 during cell death of the human breast cancer cell line MCF-7. *Mol Cell Endocrinol*. 162(1-2):151-6.

Oliveira, L. S., de Queiroz, N. M., Veloso, L. V., Moreira, T. G., Oliveira, F. S., Carneiro, M. B., Faria, A. M., Vieira, L. Q., Oliveira, S. C., Horta, M. F. (2014a). A Defective TLR4 Signaling for IFN-β Expression Is Responsible for the Innately Lower Ability of BALB/c Macrophages to Produce NO in Response to LPS as Compared to C57BL/6. *PLoS One*. 9(6):e98913.

Oliveira, T. M., de Faria, F. R., de Faria, E. R., Pereira, P. F., Franceschini, S. C., Priore, S. E. (2014b). [Nutritional status, metabolic changes and white blood cells in adolescents.] *Rev Paul Pediatr*. 32(4):351-9.

Olson, T. S., Ley, K. (2002). Chemokines and chemokine receptors in leukocyte trafficking. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology.* 283:R7-R28.

Oltmanns, U., Chung, K. F., Walters, M., John, M., Mitchell, J. A. (2005). Cigarette smoke induces IL-8, but inhibits eotaxin and RANTES release from airway smooth muscle. *Respir Res*. 6:74.

O'Neill, G. P., Mancini, J. A., Kargman, S., Yergey, J., Kwan, M. Y., Falgueyret, J. P., Abramovitz, M., Kennedy, B. P., Ouellet, M., Cromlish, W. (1994). Overexpression of human prostaglandin G/H synthase-1 and -2 by recombinant vaccinia virus: inhibition by nonsteroidal anti-inflammatory drugs and biosynthesis of 15-hydroxyeicosatetraenoic acid. *Molecular pharmacology*. 45(2):245-54.

Ordentlich, P., Yan, Y., Zhou, S., Heyman, R. A. (2003). Identification of the antineoplastic agent 6-mercaptopurine as an activator of the orphan nuclear hormone receptor Nurr1. *J Biol Chem*. 278(27):24791–24799.

Ormerod, M. G., Sun, X.-M., Snowden, R. T., Davies, R., Fearnhead, H., Cohen, G. M. (1993). Increased membrane permeability of apoptotic thymocytes: a flow cytometric study. *Cytometry*. 14:595-602.

O'Shaughnessy, T. C., Ansari, T. W., Barnes, N. C., Jeffery, P. K. (1997). Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *Am J Respir Crit Care Med*. 155(3):852-7.

Ottonello, L., Gonella, R., Dapino, P., Sacchetti, C., Dallegri, F. (1998). Prostaglandin E2 inhibits apoptosis in human neutrophilic polymorphonuclear leukocytes: role of intracellular cyclic AMP levels. *Exp Hematol*. 26(9):895-902.

Overbeek, S. A., Braber, S., Koelink, P. J., Henricks, P. A., Mortaz, E., LoTam Loi, A. T., Jackson, P. L., Garssen, J., Wagenaar, G. T., Timens, W., Koenderman, L., Blalock, J. E., Kraneveld, A. D., Folkerts, G. (2013). Cigarette smoke-induced collagen destruction; key to chronic neutrophilic airway inflammation? *PLoS ONE*. 8(1):55612.

Pace, E., Giarratano, A., Ferraro, M., Bruno, A., Siena, L., Mangione, S., Johnson, M., Gjomarkaj, M. (2011). TLR4 upregulation underpins airway neutrophilia in smokers with chronic obstructive pulmonary disease and acute respiratory failure. *Hum Immunol*. 72(1):54-62.

Pachot, A., Blond, J. L., Mougin, B., Miossec, P. (2004). Peptidylpropyl isomerase B (PPIB): a suitable reference gene for mRNA quantification in peripheral whole blood. *J Biotechnol*. 114(1-2):121-4.

Panzner, P., Lafitte, J. J., Tsicopoulos, A., Hamid, Q., Tulic, M. K. (2003). Marked up-regulation of T lymphocytes and expression of interleukin-9 in bronchial biopsies from patients with chronic bronchitis with obstruction. *Chest*. 124: 1909-1915.

Park, K., Nowyhed, H., Hedrick, C. (2013). Nr4a1 (Nur77) regulates B cell survival and activation. (P1453). *J Immunol*. 190:174.9.

Parker, L. C., Whyte, M. K., Dower, S. K., Sabroe, I. (2005). The expression and roles of Toll-like receptors in the biology of the human neutrophil. *J Leukoc Biol*. 77(6):886-92.

Parker, L. C., Whyte, M. K., Vogel, S. N., Dower, S. K., Sabroe, I. (2004). Toll-like receptor (TLR)2 and TLR4 agonists regulate CCR expression in human monocytic cells. *J Immunol*. 172(8):4977-86.

Parkkonen, J., Hasala, H., Moilanen, E., Giembycz, M. A., Kankaanranta, H. (2008). Phosphodiesterase 4 inhibitors delay human eosinophil and neutrophil apoptosis in the absence and presence of salbutamol. *Pulm Pharmacol Ther*. 21(3):499-506.

Parvathenani, L. K., Buescher ,E. S., Chacon-Cruz, E., Beebe, S. J. (1998). Type I cAMP-dependent protein kinase delays apoptosis in human neutrophils at a site upstream of caspase-3. *J Biol Chem*. 273(12):6736-43.

Pauwels, R. A., Buist, A. S., Calverley, P. M., Jenkins, C. R., Hurd, S. S. (2001). GOLD Scientific Committee. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 163(5): 1256 – 1276.

Pavlovic, S., Du, B., Sakamoto, K., Khan, K. M., Natarajan, C., Breyer, R. M., Dannenberg, A. J., Falcone, D. J. (2006). Targeting Prostaglandin E2 Receptors as an Alternative Strategy to Block Cyclooxygenase-2-dependent Extracellular Matrix-induced Matrix Metalloproteinase-9 Expression by Macrophages. *J. Biol. Chem*. 281:3321–3328.

Pavord, I. D., Tattersfield, A. E. (1995). Bronchoprotective role for endogenous prostaglandin E2. *Lancet*. 344:436-438.

Pearen, M. A., Ryall, J. G., Maxwell, M. A., Ohkura, N., Lynch, G. S., Muscat, G. E. (2006). The orphan nuclear receptor, NOR-1, is a target of beta-adrenergic signaling in skeletal muscle. *Endocrinology*. 147:5217–5227.

Pei, L., Castrillo, A., Chen, M., Hoffmann, A., Tontonoz, P. (2005). Induction of NR4A orphan nuclear receptor expression in macrophages in response to inflammatory stimuli. *J Biol Chem*. 280:29256–29262.

Pei, L., Castrillo, A., Tontonoz, P. (2006). Regulation of macrophage inflammatory gene expression by the orphan nuclear receptor Nur77. *Mol Endocrinol*. 20(4):786-94.

Perng, D. W., Huang, H. Y., Chen, H. M., Lee, Y. C., Perng, R. P. (2004). Characteristics of airway inflammation and bronchodilator reversibility in COPD: a potential guide to treatment. *Chest*. 126(2): 375–381.

Perttunen, H., Moilanen, E., Zhang, X., Barnes, P. J., Kankaanranta, H. (2008). Beta2-agonists potentiate corticosteroid-induced neutrophil survival. *COPD*. 5(3):163-9.

Pesci, A., Rossi, G. A., Bertorelli, G., Aufiero, A., Zanon, P., Olivieri, D. (1994). Mast cells in the airway lumen and bronchial mucosa of patients with chronic bronchitis. *Am J Respir Crit Care Med*. 149(5):1311–1316.

Pesci, A., Balbi, B., Majori, M., Cacciani, G., Bertacco, S., Alciato, P., Donner, C. F. (1998). Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur Respir J*. 12(2):380-6.

Pettersen, C. A., Adler, K. B. (2002). Airways inflammation and COPD: epithelialneutrophil interactions. *Chest*. 121(Suppl):142S-150S.

Pham, C. T. N. (2006). Neutrophil serine proteases: specific regulators of inflammation. *Nature Reviews Immunology*. 6:541-550.

Phillis, J. W. (2004). Adenosine and adenine nucleotides as regulators of cerebral blood flow: roles of acidosis, cell swelling, and KATP channels. *Crit Rev Neurobiol*. 16(4):237-70.

Pilette, C., Ouadrhiri, Y., Godding, V., Vaerman, J. P., Sibille, Y. (2001). Lung mucosal immunity: immunoglobulin-A revisited. *Eur Respir J*. 18:571-588.

Pillay, J., den Braber, I. Vrisekoop, N., Kwast, L. M., de Boer, R. J., Borghans, J. A. M., Tesselaar, K., Koenderman, L. (2010). *In vivo* labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood.* 116(4):625-627.

Pineault, N., Helgason, C.D., Lawrence, H.J. & Humphries, R.K. (2002) Differential expression of Hox, Meis1, and Pbx1 genes in primitive cells throughout murine hematopoietic ontogeny. *Experimental Hematology.* 30:49-57.

Pletz, M.W.R., Ioanas, M., de Roux, A., Burkhardt, O., Lode, H. (2004). Reduced spontaneous apoptosis in peripheral blood neutrophils during exacerbation of COPD. *Eur Respir J*. 23:532–537.

Pletz, M. W. R., Lode, H. (2005). Apoptosis of Circulating Neutrophils in COPD Patients. *Chest*. 127(4):1464-1465.

Pliyev, B. K., Dimitrieva, T. V., Savchenko, V. G. (2014). Diadenosine diphosphate (Ap2A) delays neutrophil apoptosis via the adenosine A2A receptor and cAMP/PKA pathway. Biochem. *Cell Biol*. 92:1–5.

Polet, H., Levine, L., (1975). Metabolism of Prostaglandins E, A, and C in Serum. *The Journal of Biological Chemistry*. 250 (2): 361-357.

Polosa, R., Blackburn, M. R. (2009). Adenosine receptors as targets for therapeutic intervention in asthma and Chronic Obstructive Pulmonary Disease. *Trends Pharmacol Sci*. 30(10): 528–535.

Poloso, N. J., Urquhart, P., Nicolaou, A., Wang, J., Woodward, D. F. (2013). PGE2 differentially regulates monocyte-derived dendritic cell cytokine responses depending on receptor usage (EP2/EP4). *Mol Immunol*. 54(3-4):284-95.

Pongracz, J., Webb, P., Wang, K., Deacon, E., Lunnand, O. J., Lord, J. M. (1999). Spontaneous Neutrophil Apoptosis Involves Caspase 3-mediated Activation of Protein Kinase C-δ. *The Journal of Biological Chemistry*. 274:37329-37334.

Porter, A. G., Jänicke, R. U. (1999). Emerging roles of caspase-3 in apoptosis. *Cell Death Differ*. 6(2):99-104.

Porter, J. C., Falzon, M., Hall, A. (2008). Polarized localization of epithelial CXCL11 in chronic obstructive pulmonary disease and mechanisms of T cell egression. *J Immunol*. 180(3):1866-77.

Pow, D., Schleiss, M. B., Kanniess, F., Taube, C., Jorres, R. A., Magnussen, H. (2001). In patients with chronic bronchitis a four week trial with inhaled steroids does not attenuate airway inflammation. *Respir. Med*. 95:115–121.

Prieto, A., Reyes, E., Bernstein, E. D., Martinez, B., Monserrat, J., Izquierdo, J. L., Callol, L., de Lucas, P., Alvarez-Sala, R., Alvarez-Sala, J. L., Villarrubia, V. G., Alvarez-Mon, M. (2001). Defective natural killer and phagocytic activities in chronic obstructive pulmonary disease are restored by glycophosphopeptical (inmunoferón). *Am J Respir Crit Care Med*. 163(7):1578-83.

Pripp, H., Stanišić, M. (2014). The Correlation between Pro- and Anti-Inflammatory Cytokines in Chronic Subdural Hematoma Patients Assessed with Factor Analysis. *PLoS One*. 9(2):e90149.

Profita, M., Giorgi, R. D., Sala, A., Bonanno, A., Riccobono, L., Mirabella, F., Gjomarkaj, M., Bonsignore, G., Bousquet, J., Vignola, A. M. (2005). Muscarinic receptors, leukotriene B4 production and neutrophilic inflammation in COPD patients. *Allergy*. 60(11):1361-9.

Profita, M., Sala, A., Bonanno, A., Riccobono, L., Ferraro, M., La Grutta, S., Albano, G. D., Montalbano, A. M., Gjomarkaj, M. (2010). Chronic obstructive pulmonary disease and neutrophil infiltration: role of cigarette smoke and cyclooxygenase products. *Am J Physiol Lung Cell Mol Physiol*. 298(2):L261-9.

Punturieri, A., Filippov, S., Allen, E., Caras, I., Murray, R., Reddy, V., Weiss, S. J. (2000). Regulation of elastinolytic cysteine proteinase activity in normal and cathepsin K-deficient human macrophages. *J Exp Med.* 192:789-800.

Rabe, K. F., Hurd, S., Anzueto, A., Barnes, P. J., Buist, S. A., Calverley, P., Fukuchi, Y., Jenkins, C., Rodriguez-Roisin, R., van Weel, C., Zielinski, J., Global Initiative for Chronic Obstructive Lung Disease. (2007). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med*. 176(6):532-55.

Ralph, J. A., McEvoy, A. N., Kane, D., Bresnihan, B., FitzGerald, O., Murphy, E. P. (2005). Modulation of orphan nuclear receptor NURR1 expression by methotrexate in human inflammatory joint disease involves adenosine A2A receptor-mediated responses. *J Immunol*. 175(1):555-65.

Randolph, G. J., Beaulieu, S., Lebecque, S., Steinman, R. M., Muller, W. A. (1998). Differentiation of Monocytes into Dendritic Cells in a Model of Transendothelial Trafficking. *Science*. 282: 480-483.

Regenhard, P., Goethe, R., Phi-van, L. (2001). Involvement of PKA, PKC, and Ca2+ in LPS-activated expression of the chicken lysozyme gene. *J Leukoc Biol*. 69(4):651-8.

Renshaw, S. A., Parmar, J. S., Singleton, V., Rowe, S. J., Dockrell, D. H., Dower, S. K., Bingle, C. D., Chilvers, E. R., Whyte, M. K. (2003). Acceleration of human neutrophil apoptosis by TRAIL. *J Immunol*. 170(2):1027-33.

Renshaw, S. A., Timmons, S. J., Eaton, V., Usher, L. R., Akil, M. Bingle, C. D., Whyte, M. K. B. (2000). Inflammatory neutrophils retain susceptibility to apoptosis mediated via the Fas death receptor. *Journal of Leukocyte Biology.* 67:662-668.

Retamales, I., Elliott, W. M., Meshi, B., Coxson, H. O., Pare, P. D., Sciurba, F. C., Rogers, R. M., Hayashi, S., Hogg, J. C. (2001). Amplification of inflammation in emphysema and its association with latent adenoviral infection. *Am J Respir Crit Care Med*. 164(3):469–473.

Ricciotti, E., FitzGerald, G. A. (2011). Prostaglandins and Inflammation. *Arterioscler Thromb Vasc Biol*. 31(5): 986–1000.

Richards, G. A., Theron, A. J., Van der Merwe, C. A., Anderson, R. (1989). Spirometric abnormalities in young smokers correlate with increased chemiluminescence responses of activated blood phagocytes. *Am Rev Respir Dis*. 139:181-187.

Rigamonti, E., Chinetti-Gbaguidi, G., Staels, B. (2008). Regulation of Macrophage Functions by PPAR-, PPAR-, and LXRs in Mice and Men. *Arterioscler Thromb Vasc Biol*. 28:1050-1059.

Riise, G. C., Ahlstedt, S., Larsson, S., Enander, I., Jones, I., Larsson, P., Andersson, B. (1995). Bronchial inflammation in chronic bronchitis assessed by measurement of cell products in bronchial lavage fluid. *Thorax*. 50(4):360-5.

Risso, A. (2000) Leukocyte antimicrobial peptides: multifunctional effector molecules of innate immunity. *Journal of Leukocyte Biology*, 68, 785-792.

Rivero, J. A., Adunyah, S. E. (1998). Sodium Butyrate Stimulates PKC Activation and Induces Differential Expression of Certain PKC Isoforms During Erythroid Differentiation. *Biochem. Biophys. Res.Commun.* 248:664 – 668.

Roberson, E. D., English, J. D., Adams, J. P., Selcher, J. C., Kondratick, C., Sweatt, J. D. (1999). The Mitogen-Activated Protein Kinase Cascade Couples PKA and PKC to cAMP Response Element Binding Protein Phosphorylation in Area CA1 of Hippocampus. *The Journal of Neuroscience*. 19(11):4337–4348.

Roberts, A. W. (2005). G-CSF: A key regulator of neutrophil production, but that's not all! *Growth Factors*. 23(1):33-41.

Roberts, C. M., Lowe, D., Bucknall, C. E., Ryland, I., Kelly, Y., Pearson, M. G. (2002). Clinical audit indicators of outcome following admission to hospital with acute exacerbation of chronic obstructive pulmonary disease. *Thorax*. 57(2):137-41.

Rose, T. M., Reilly, C. A., Deering-Rice, C. E., Brewster, C., Brewster, C. (2014). Inhibition of FAAH, TRPV1, and COX2 by NSAID-serotonin conjugates. *Bioorg Med Chem Lett*. 24(24):5695-8.

Roshan-Moniri, M., Hsing, M., Butler, M. S., Cherkasov, A., Rennie, P. S. (2014). Orphan nuclear receptors as drug targets for the treatment of prostate and breast cancers. *Cancer Treat Rev*. 40(10):1137-52.

Rossi, A. G., Cousin, J. M., Dransfield, I., Lawson, M. F., Chilvers, E. R., Haslett, C. (1995). Agents that elevate cAMP inhibit human neutrophil apoptosis. *Biochem Biophys Res Commun*. 217(3):892-9.

Rossi, A., Khirani, S., Cazzola, M. (2008). Long-acting β2-agonists (LABA) in chronic obstructive pulmonary disease: efficacy and safety. *Int J Chron Obstruct Pulmon Dis*. 3:521–529.

Rossol, M., Heine, H., Meusch, U., Quandt, D., Klein, C., Sweet, M. J., Hauschildt, S. (2011). LPS-induced cytokine production in human monocytes and macrophages. *Crit Rev Immunol*. 31(5):379-446.

Rundhaug, J. E., Simper, M. S., Surh, I., Fischer, S. M. (2011). The role of the EP receptors for prostaglandin E2 in skin and skin cancer. *Cancer Metastasis Rev*. 30(3-4):465-80.

Rutgers, S. R., Postma, D. S., ten Hacken, N. H., Kauffman, H. F., van Der Mark, T. W., Koëter, G. H., Timens, W. (2000). Ongoing airway inflammation in patients with COPD who do not currently smoke. *Thorax*. 55(1):12-8.

Rytila, P., Plataki, M., Bucchieri, F., Uddin, M., Nong, G., Kinnula, V. L., Djukanovic, R. (2006). Airway neutrophilia in COPD is not associated with increased neutrophil survival. *Eur Respir J*. 28: 1163–1169.

Saalbach, A., Janik, T., Busch, M., Herbert, D., Anderegg, U., Simon, J. C. (2015). Fibroblasts support migration of monocyte-derived dendritic cells by secretion of PGE2 and MMP-1. *Exp Dermatol*. 24(8):598-604.

Saba, S., Soong, G., Greenberg, S. & Prince, A. (2002) Bacterial stimulation of epithelial G-CSF and GM-CSF expression promotes PMN survival in CF airways. *American Journal of Respiratory Cell and Molecular Biology*, 27, 561-567.

Sabroe, I., Jones, E.C., Usher, L.R., Whyte, M.K.B. & Dower, S.K. (2002a) Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: A critical role for monocytes in leukocyte lipopolysaccharide responses. *Journal of Immunology*, 168, 4701-4710.

Sabroe, I., Lloyd, C. M., Whyte, M. K. B., Dower, S. K., Williams, T. J., Pease, J. E. (2002b). Chemokines, innate and adaptive immunity, and respiratory disease. *Eur Respir J*. 19(2):350–355.

Sabroe, I., Prince, L. R., Jones, E. C., Horsburgh, M. J., Foster, S. J., Vogel, S. N., Dower, S. K., Whyte, M. K. (2003). Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. *J Immunol*. 170(10):5268-75.

Sabroe, I., Dower, S. K., Whyte, M. K. (2005). The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis. *Clin Infect Dis*. 41 Suppl 7:S421-6.

Sabroe, I., Parker, L. C., Calverley, P. M. A., Dower, S. K., Whyte, M. K. B. (2007). Pathological networking: a new approach to understanding COPD. *Thorax*. 62(8): 733–738.

Sabroe, I., Whyte, M. K. B. (2007). Incapacitating the immune system in cystic fibrosis. *Nature Medicine*. 13:1417–1418.

Sabroe, I., Postma, D., Heijink, I., Dockrell, D. H. (2013). The yin and the yang of immunosuppression with inhaled corticosteroids. *Thorax*. 68:1085-1087.

Saetta, M., Di Stefano, A., Maestrelli, P., Ferraresso, A., Drigo, R., Potena, A., Ciaccia, A., Fabbri, L. M. (1993). Activated T-lymphocytes and macrophages in bronchial mucosa of subjects with chronic bronchitis. *Am Rev Respir Dis.* 147(2):301-6.

Saetta, M., DiStefano, A., Maestrelli, P., Graziella, T., Rugieri, M. P., Roggeri, A., Calcagni, P., Mapp, C. E., Ciaccia, A., Fabbri, L. M. (1994). Airway eosinophilia in chronic bronchitis during exacerbations. *Am J Respir Crit Care Med*. 150:1646-1652.

Saetta, M., Mariani, M., Panina-Bordignon, P., Turato, G., Buonsanti, C., Baraldo, S., Bellettato, C. M., Papi, A., Corbetta, L., Zuin, R., Sinigaglia, F., Fabbri, L. M. (2002). Increased expression of the chemokine receptor CXCR3 and its ligand CXCL10 in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 165:1404-1409.

Safe, S., Jin, U.-H., Hedrick, E., Reeder, A., Lee, S.-O. (2014). Minireview: Role Of Orphan Nuclear Receptors in Cancer and Potential as Drug Targets. *Mol Endocrinol*. 28(2):157–172.

Saffar, A. S., Ashdown, H., Gounni, A. S. (2011). The Molecular Mechanisms of Glucocorticoids-Mediated Neutrophil Survival. *Current Drug Targets*. 12: 556-562.

Salminen, A., Tapiola, T., Korhonen, P., Suuronen, T. (1998). Neuronal Apoptosis Induced by Histone Deacetylase Inhibitors. *Mol. Brain Res*. 61:203-206.

Salpeter, S. R., Ormiston, T. M., Salpeter, E. E. (2004). Cardiovascular effects of β-agonists in patients with asthma and COPD: a meta-analysis. *Chest*. 125:2309–2321.

Sandig, H., Pease, J. E., Sabroe, I. (2007). Contrary prostaglandins: the opposing roles of PGD2 and its metabolites in leukocyte function. *Journal of Leukocyte Biology*. 81(2):372-382.

Saraste A. (1999). Morphologic criteria and detection of apoptosis. *Herz*. 24(3):189-95.

Saucedo-Cardenas, O., Quintana-Hau, J. D., Le, W. D., Smidt, M. P., Cox, J. J., De Mayo, F., Burbach, J. P., Conneely, O. M. (1998). Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc Natl Acad Sci U S A*. 95(7):4013-8.

Savill, J. S., Wyllie, A. H., Henson, J. E., Wolport, M. J., Henson, P. M., Haslett, C. (1989). Macrophage phagocytosis of aging neutrophils in inflammationprogrammed cell death in the neutrophil leads to its recognition by macrophages. *J. Clin. Invest*. 83:865–875.

Savill, J. (1997a). Apoptosis in resolution of inflammation. *J Leukoc Biol.* 61:375–380.

Savill, J. (1997b). Recognition and phagocytosis of cells undergoing apoptosis. *Br. Med. Bull*. 53(3):491-508.

Savill, J., Fadok, V. (2000). Corpse clearance defines the meaning of cell death. *Nature*. 407:784–788.

Savill, J., Dransfield, I., Gregory, C., Haslett, C. (2002). A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat. Rev. Immunol*. 2:965-975. Review.

Scapini, P., Lapinet-Vera, J. A., Gasperini, S., Calzetti, F., Bazzoni, F., Cassatella, M. A. (2000). The neutrophil as a cellular source of chemokines. *Immunol Rev.* 177:195-203. Review.

Schiffmann, E., Corcoran, B. A., Whal, S. M. (1975). N-formylmethionyl peptides as chemoattractants for leucocytes. *Proceedings of the National Academy of Sciences.* 72:1059-1062.

Schlegel, W., Greep, R. O. (1976). Kinetic studies on 15-hydroxyprostaglandin dehydrogenase from human placenta. *Advances in prostaglandin and thromboxane research*. 1:159-62.

Schmid, I., Uitenbogaart, C. H., Giorgi, J.V. (1994). Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. *Cytometry.* 15:12-20.

Schmidt, L. M., Belvisi, M. G., Bode, K. A., Bauer, J., Schmidt, C., Suchy, M. T., Tsikas, D., Scheuerer, J., Lasitschka, F., Gröne, H. J., Dalpke, A. H. (2011). Bronchial epithelial cell-derived prostaglandin E2 dampens the reactivity of dendritic cells. *J Immunol*. 186(4):2095-105.

Schmidt-Ioanas, M., Pletz, M. W. R., de Roux, A., Lode, H. (2006). Apoptosis of peripheral blood neutrophils in COPD exacerbation does not correlate with serum cytokines. *Respiratory Medicine*. 100(4): 639–647.

Schwede, F., Christensen, A., Liauw, S., Hippe, T., Kopperud, R., Jastorff, B., Døskeland, S. O. (2000a). 8-Substituted cAMP analogues reveal marked differences in adaptability, hydrogen bonding, and charge accommodation between homologous binding sites (AI/AII and BI/BII) in cAMP kinase I and II. *Biochemistry*. 39(30):8803-12.

Schwede, F., Maronde, E., Genieser, H.-G., Jastorff, B. (2000b). Cyclic nucleotide analogs as biochemical tools and prospective drugs. *Pharmacol Ther*. 87(2-3):199-226.

Scott, T., Mercer, E. I. (1995). *Concise Encyclopedia biochemistry and molecular biology*. Spektrum Akademischer Verlag, Berlin.

Scott, J. W., Hawley, S. A., Green, K. A., Anis, M., Stewart, G., Scullion, G. A., Norman, D. G., Hardie, D. G. (2004). CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *J. Clin. Invest.* 113:274-284.

Seebach, J. D., Morant, R., Rüegg, R., Seifert, B., Fehr, J. (1997). The diagnostic value of the neutrophil left shift in predicting inflammatory and infectious disease. *Am J Clin Pathol*. 107(5):582-91.

Segura-Valdez, L., Pardo, A., Gaxiola, M., Uhal, B. D., Becerril, C., Selman, M. (2000). Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD. *Chest*. 117:684–694.

Sekiya, T., Kashiwagi, I., Yoshida, R., Fukaya, T., Morita, R., Kimura, A., Ichinose, H., Metzger, D., Chambon, P., Yoshimura, A. (2013). Nr4a receptors are essential for thymic regulatory T cell development and immune homeostasis. *Nat Immunol*. 14(3):230-7.

Selman, M., Cisneros-Lira, J., Gaxiola, M., Ramírez, R., Kudlacz, E. M., Mitchell, P. G., Pardo, A. (2003). Matrix metalloproteinases inhibition attenuates tobacco smoke-induced emphysema in guinea pigs. *Chest*. 123(5):1633–1641.

Servant, G., Weiner, O. D., Herzmark, P. Balla, T., Sedat, J. W., Bourne, H. R. (2000). Polarization of Chemoattractant Receptor Signaling During Neutrophil Chemotaxis. *Science*. 287(5455):1037–1040.

Shamsuddin, M., Chen, E., Anderson, J., Smith, L. J. (1997). Regulation of leukotriene and platelet-activating factor synthesis in human alveolar macrophages. *J Lab Clin Med*. 130:615-626.

Shapiro, S. D., Goldstein, N. M., McGarry Houghton, A., Kobayashi, D. K., Kelley, D., Belaaouaj, A. (2003). Neutrophil Elastase Contributes to Cigarette Smoke-Induced Emphysema in Mice. *Am J Pathol*. 163(6): 2329–2335.

Sheibanie, A. F., Tadmori, I., Jing, H., Vassiliou, E., Ganea, D. (2004). Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells. *FASEB J*. 18:1318-1320.

Sheibanie, A. F., Yen, J. H., Khayrullina, T., Emig, F., Zhang, M., Tuma, R., Ganea, D. (2007). The proinflammatory effect of prostaglandin E2 in experimental inflammatory bowel disease is mediated through the IL-23-->IL-17 axis. *J Immunol*. 178(12):8138-47.

Shi, Y. (2007). Orphan Nuclear Receptors in Drug Discovery. *Drug Discov Today*. 12(11-12):440–445.

Shigeishi, H., Higashikawa, K., Hatano, H., Okui, G., Tanaka, F., Tran, T. T., Rizqiawan, A., Ono, S., Tobiume, K., Kamata, N. (2011). PGE_2 targets squamous cell carcinoma cell with the activated epidermal growth factor receptor family for survival against 5-fluorouracil through NR4A2 induction. *Cancer Lett*. 307(2):227-36.

Shimamura, M., Zhou, P., Casolla, B., Qian, L., Capone, C., Kurinami, H., Iadecola, C., Anrather, J. (2013). Prostaglandin E2 type 1 receptors contribute to neuronal apoptosis after transient forebrain ischemia. *J Cereb Blood Flow Metab*. 33(8):1207-14.

Silva, M. T. (2010). When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. *Journal of Leukocyte Biology*. 87:93-106.

Simon, H.-U. (2003). Neutrophil apoptosis pathways and their modifications in inflammation. *Immunological Reviews*. 193(1):101-110.

Sin, D. D., Lacy, P., York, E., Man, S. F. (2004). Effects of fluticasone on systemic markers of inflammation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 170:760-765.

Singh, D., Brooks, J., Hagan, G., Cahn, A., O'Connor, B. J. (2008). Superiority of 'triple' therapy with salmeterol/fluticasone propionate and tiotropium bromide versus individual components in moderate to severe COPD. *Thorax*. 63:592–598.

Singh, D., Edwards, L., Singer, R. T., Rennard, S. (2010). Sputum neutrophils as a biomarker in COPD: findings from the ECLIPSE study. *Respiratory Research.* 11:77.

Sjöholm, A.; Diabetes 46, 1141 - 1147 (1997): "Glucose Stimulates Islet β-Cell Mitogenesis Through GTP Binding Proteins and by Protein Kinase C-Dependent Mechanisms"

Skalhegg, B.S. & Tasken, K. (2000) Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation, and subcellular localization of subunits of PKA. *Frontiers in Bioscience*, 5, D678-D693.

Slee, E. A., Adrain, C., Martin, S. J. (2001). Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J Biol Chem*. 276(10):7320-6.

Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H.-G., Reed, J. C., Nicholson, D. W., Alnemri, E. S., Green, D. R., Martin, S. J. (1999). Ordering the Cytochrome c–initiated Caspase Cascade: Hierarchical Activation of Caspases-2, -3, -6, -7, -8, and -10 in a Caspase-9–dependent Manner. *Cell Biol.* 144(2): 281-292.

Smith, C. W., Marlin, S. D., Rothlein, R., Toman, C., & Anderson, D. C. (1989). Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils *in vitro*. *Journal of Clinical Investigation.* 83: 2008-2017.

Smyth, E. M., Grosser, T., Wang, M., Yu, Y., FitzGerald, G. A. (2009). Prostanoids in health and disease. *J Lipid Res*. 50:423–428.

Snider, G. L., Lucey, E. C., Stone, P. J. (1986). Animal Models of Emphysema. *American Review of Respiratory Disease*. 133(1):149-169.

Soldatenkov, V. A., Prasad, S., Voloshin, Y., Dritschilo, A. (1998). Sodium Butyrate Induces Apoptosis and Accumulation of Ubiquitinated Proteins in Human Breast Carcinoma Cells. *Cell Death Differentiation*. 5:307–312.

Soler, P., Moreau, A., Basset, F., Hance, A. J. (1989). Cigarette smoking-induced changes in the number and differentiated state of pulmonary dendritic cells/Langerhans cells. *Am Rev Respir Dis*. 139:1112-1117.

Soler-Rodriguez, A. M., Zhang, H., Lichenstein, H. S., Qureshi, N., Niesel, D. W., Crowe, S. E., Peterson, J. W., Klimpel, G. R. (2000). Neutrophil Activation by Bacterial Lipoprotein Versus Lipopolysaccharide: Differential Requirements for Serum and CD141. *The Journal of Immunology*. 164:2674-2683.

Sommerhoff, C. P., Nadel, J. A., Basbaum, C. B., Caughey, G. H. (1990). Neutrophil elastase and cathepsin G stimulate secretion from cultured bovine airway gland serous cells. *J Clin Investig*. 85: 682-689.

Sousa, L. P., Lopes, F., Silva, D. M., Tavares, L. P., Vieira, A. T., Rezende, B. M., Carmo, A. F., Russo, R. C., Garcia, C. C., Bonjardim, C. A., Alessandri, A. L., Rossi, A. G., Pinho, V., Teixeira, M. M. (2010). PDE4 inhibition drives resolution of neutrophilic inflammation by inducing apoptosis in a PKA-PI3K/Akt-dependent and NF-kappaB-independent manner. *J Leukoc Biol*. 87(5):895-904.

Sparrow, D., Glynn, R. J., Cohen, M., Weiss, S. T. (1984). The relationship of the peripheral leukocyte count and cigarette smoking to pulmonary function among adult men. *Chest.* 86: 383-386.

Spertini, O., Kansas, G. S., Munro, J. M., Griffin, J. D., Tedder, T. F. (1991). Regulation of leukocyte migration by activation of the leukocyte adhesion molecule-1 (LAM-1) selectin. *Nature.* 349(6311):691-4.

Stadel, J. M., Nakada, M. T., Crooke, S. T. (1987). Molecular Mechanisms of ß-Adrenergic Receptor Regulation. *Int. Congr. Ser.- Exerpta Med*. 750 (17).

Stanescu, D., Sanna, A., Veriter, C., Kostianev, S., Callagni, P. G., Fabbri, L. M., Maestrelli, P. (1996). Airways obstruction, chronic expectoration and rapid decline in FEV1 in smokers are associated with increased levels of sputum neutrophils. *Thorax*. 51: 267-271.

Stanojkovic, I., Kotur-Stevuljevic, J., Milenkovic, B., Spasic, S., Vujic, T., Stefanovic, A., Llic, A., Ivanisevic, J. (2011). Pulmonary function, oxidative stress and inflammatory markers in severe COPD exacerbation. *Respir. Med*. 105(Suppl. 1), S31–S37.

Stewart, A. G., Tomlinson, P. R., Wilson, J. W. (1997). β2-Adrenoceptor agonist-mediated inhibition of human airway smooth muscle cell proliferation: importance of the duration of β2-adrenoceptor stimulation. *Br J Pharmacol*. 121(3): 361–368.

Stork, P. J., Schmitt, J. M. (2002). Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends Cell Biol*. 12(6):258-66.

Strange, C., Beiko, T. (2015). Treatment of Alpha-1 Antitrypsin Deficiency. *Semin Respir Crit Care Med*. 36(4):470-7.

Strassburg, A., Luers, A., Dalhoff, K. (2010). Decreased apoptosis of pulmonary PMN in COPD patients with community-acquired pneumonia. *Clin Respir J*. 4(2):111-9.

Strathmann, M.P. & Simon, M.I. (1991) G-alpha-12 and G-alpha-13 subunits define a 4th class of G-protein alpha subunits. *Proceedings of the National Academy of Sciences of the United States of America*. 88:5582-5586.

Sugimoto, Y., Namba, T., Honda, A., Hayashi, Y., Negishi, M., Ichikawa, A., Narumiya, S. (1992). Cloning and expression of a cDNA for mouse prostaglandin E receptor EP3 subtype. *J Biol Chem*. 267(10):6463-6.

Sugimoto, Y., Narumiya, S. (2007). Prostaglandin E Receptors. *J Biol Chem*. 282(16):11613-7.

Sullivan, G. W. (2003). Adenosine A2A receptor agonists as anti-inflammatory agents. *Curr Opin Investig. Drugs*. 4:1313–1319.

Sunahara, R.K., Dessauer, C.W. & Gilman, A.G. (1996) Complexity and diversity of mammalian adenylyl cyclases. *Annual Review of Pharmacology and Toxicology*. 36:461-480.

Suratt, B. T., Young, S. K., Liber, J., Nick, J. A., Henson, P. M., Worthen, G. S. (2001). Neutrophil maturation and activation determine anatomic site of clearance from circulation. *AJP – Lung Physiol*. 281(4): L913-L921.

Suratt, B. T., Petty, J. M., Young, S. K., Malcolm, K. C., Lieber, J. G., Nick, J. A., Gonzalo, J.-A., Henson, P. M., Worthen, G. S. (2004). Role of the CXCR4/SDF-1 chemokine axis in circulating neutrophil homeostasis. *Blood*. 104(2): 565-71.

Taha, R., Olivenstein, R., Utsumi, T., Ernst, P., Barnes, P. J., Rodger, I. W., Giaid, A. (2000). Prostaglandin H synthase 2 expression in airway cells from patients with asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 161:636-640.

Tai, H. H., Ensor, C. M., Tong, M., Zhou, H., Yan, F. (2002). Prostaglandin catabolizing enzymes. *Prostaglandins Other Lipid Mediat*. 68-69:483–493.

Takizawa, H., Tanaka, M., Takami, K., Ohtoshi, T., Ito, K., Satoh, M., Okada, Y., Yamasawa, F., Nakahara, K., Umeda, A. (2001). Increased expression of transforming growth factor-β1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD). *Am J Respir Crit Care Med*. 163:1476-1483.

Takubo, Y., Guerassimov, A., Ghezzo, H., Triantafillopoulos, A., Bates, J. H., Hoidal, J. R., Cosio, M. G. (2002). α1-Antitrypsin determines the pattern of emphysema and function in tobacco smoke-exposed mice: parallels with human disease. *Am J Respir Crit Care Med*. 166:1596-1603.

Talpain, E., Armstrong, R. A., Coleman, R. A., Vardey, C. J. (1995). Characterization of the PGE receptor subtype mediating inhibition of superoxide production in human neutrophils. *Br J Pharmacol*. 114(7):1459-65.

Tamaoki, J., Chiyotani, A., Kobayashi, S., Noritaka, T., Kanemura, T., Takizawa, T. (1992). Effect of indomethacin on bronchorrhea with chronic bronchitis, diffuse panbronchiolitis or bronchiectasis. *Am Rev Respir Dis*. 145:548-552.

Tang, Y., Chen, Y., Jiang, H., Robbins, G. T., Nie, D. (2011). G-protein-coupled receptor for short-chain fatty acids suppresses colon cancer. *Int J Cancer*. 128(4):847-56.

Tanino, M., Betsuyaku, T., Takeyabu, K., Tanino, Y., Yamaguchi, E., Miyamoto, K., Nishimura, M. (2002). Increased levels of interleukin-8 in BAL fluid from smokers susceptible to pulmonary emphysema. *Thorax*. 57: 405-411.

Tarek, M. (2005). Membrane Electroporation: A Molecular Dynamics Simulation. *Biophys J*. 88(6): 4045–4053.

Taussig, R., Gilman, A. G. (1995). Mammalian membrane-bound adenylyl cyclases. *J Biol Chem*. 270(1):1-4.

Taylor, A. E., Finney-Hayward, T. K., Quint, J. K., Thomas, C. M., Tudhope, S. J., Wedzicha, J. A., Barnes, P. J., Donnelly, L. E. (2010). Defective macrophage phagocytosis of bacteria in COPD. *Eur Respir J*. 35(5):1039-47.

Tecchio, C., Micheletti, A., Cassatella, M. A. (2014). Neutrophil-derived cytokines: facts beyond expression. *Front Immunol*. 5:508.

Terashima, T., Wiggs, B., English, D., Hogg, J. C., van Eeden, S. F. (1997). Phagocytosis of small carbon particles (PM10) by alveolar macrophages stimulates the release of polymorphonuclear leukocytes from bone marrow. *Am J Respir Crit Care Med*. 155: 1441-1447.

Tetley, T. D. (1993). New perspectives on basic mechanisms in lung disease. 6. Proteinase imbalance: its role in lung disease. *Thorax*. 48(5):560–5.

Theilgaard-Mönch, K., Jacobsen, L. C., Borup, R., Rasmussen, T., Bjerregaard, M. D., Nielsen, F. C., Cowland, J. B., Borregaard, N. (2005). The transcriptional program of terminal granulocytic differentiation. *Blood*. 105,1785–1796.

Thimmulappa, R. K., Gang, X., Kim, J. H., Sussan, T. E., Witztum, J. L., Biswal, S. (2012). Oxidized phospholipids impair pulmonary antibacterial defenses: evidence in mice exposed to cigarette smoke. *Biochem. Biophys. Res. Commun*. 426(2):253–259.

Thompson, A. A., Elks, P. M., Marriott, H. M., Eamsamarng, S., Higgins, K. R., Lewis, A., Williams, L., Parmar, S., Shaw, G., McGrath, E. E., Formenti, F., Van Eeden, F. J., Kinnula, V. L., Pugh, C. W., Sabroe, I., Dockrell, D. H., Chilvers, E. R., Robbins, P. A., Percy, M. J., Simon, M. C., Johnson, R. S., Renshaw, S. A., Whyte, M. K., Walmsley, S. R. (2014). Hypoxia-inducible factor 2α regulates key neutrophil functions in humans, mice, and zebrafish. Blood. 123(3):366-76.

Thoren, S., Weinander, R., Saha, S., Jegerschold, C., Pettersson, P. L., Samuelsson, B., Hebert, H., Hamberg, M., Morgenstern, R., Jakobsson, P. J. (2003). Human microsomal prostaglandin E synthase-1: purification, functional characterization, and projection structure determination. *The Journal of biological chemistry*. 278(25):22199-209.

Thorsteinsdottir, U., Mamo, A., Kroon, E., Jerome, L., Bijl, J., Lawrence, H. J., Humphries, K., Sauvageau, G. (2002) Overexpression. of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. *Blood*. 99:121-129.

Tibbetts, M. D., Zheng, L. X., Lenardo, M. J. (2003) The death effector domain protein family: regulators of cellular homeostasis. *Nature Immunology*. 4:404-409.

Tilley, S. L., Coffman, T. M., Koller, B. H. (2001). Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. *J Clin Invest*. 108:15–23.

Toews, G. B. (2005). Impact of bacterial infections on airway diseases. *Eur. Respir. Rev.* 14(95):62-68. Review.

Traves, S. L., Culpitt, S. V., Russell, R. E., Barnes, P. J., Donnelly, L. E. (2002). Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. *Thorax*. 57:590–5.

Tsukamoto, H., Fukudome, K., Takao, S., Tsuneyoshi, N., Ohta, S., Nagai, Y., Ihara, H., Miyake, K., Ikeda, Y., Kimoto, M. (2013). Reduced surface expression of TLR4 by a V254I point mutation accounts for the low lipopolysaccharide responder phenotype of BALB/c B cells. *J Immunol*. 190(1):195-204.

Tuschl, T. (2002). Expanding small RNA interference. *Nature Biotechnology*. 20:446–448.

Ulcar, R., Peskar, B. A., Schuligoi, R., Heinemann, A., Kessler, H. H., Santner, B. I., Amann, R. (2004). Cyclooxygenase inhibition in human monocytes increases endotoxin-induced TNFα without affecting cyclooxygenase-2 expression. *European Journal of Pharmacology*. 501(1-3):9–17.

Ulcar, R., Schuligoi, R., Heinemann, A., Santner, B., Amann, R. (2003). Inhibition of prostaglandin biosynthesis in human endotoxin-stimulated peripheral blood monocytes: effects of caffeine. *Pharmacology*. 67(2):67-71.

Usher, L. R., Lawson, R. A., Geary, I., Taylor, C. J., Bingle, C. D., Taylor, G. W., Whyte, M. K. B.(2002). Induction of neutrophil apoptosis by the Pseudomonas aeruginosa exotoxin pyocyanin: a potential mechanism of persistent infection. *Journal of Immunology.* 168:1861-1868.

van den Berg, J. M., Weyer, S., Weening, J. J., Roos, D., Kuijpers, T. W. (2001). Divergent effects of tumor necrosis factor {alpha} on apoptosis of human neutrophils. *Journal of Leukocyte Biology*. 69:467-473.

Van den Steen, P. E., Proost, P., Wuyts, A., Van Damme, J., Opdenakker, G. (2000). Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-a and leaves RANTES and MCP-2 intact. *Blood*. 96(8):2673–2681.

van der Pouw Kraan, T. C., Boeije, L. C., Smeenk, R. J., Wijdenes, J., Aarden, L. A. (1995). Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. *J Exp Med*. 181:775.

Van Engeland, M., Nieland, L. J. W., Ramaekerss, F. C. S., Schutte, B., Reutelingsperger, C. P. M. (1998). Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry*. 31:1-9.

Van Hooijdonk, C. A., Glade, C. P., Van Erp, P. E. (1994). TO-PRO-3 iodide: a novel HeNe laser-excitable DNA stain as an alternative for propidium iodide in multiparameter flow cytometry. *Cytometry*. 17(2):185-9.

van Raam, B. J., Drewniak, A., Groenewold, V., van den Berg, T. K., Kuijpers, T. W. (2008). Granulocyte colony-stimulating factor delays neutrophil apoptosis by inhibition of calpains upstream of caspase-3. *Blood*. 112:2046-2054.

Vandivier, R. W., Fadok, V. A., Hoffmann, P. R., Bratton, D. L., Penvari, C., Brown, K. K., Brain, J. D., Accurso, F. J., Henson, P. M. (2002). Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. *J Clin Invest*. 109(5):661-70.

Vane, J. R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol*. 231:232–235.

Varani, K., Caramori, G., Vincenzi, F., Adcock, I., Casolari, P., Leung, E., MacLennan, S., Gessi, S., Morello, S., Barnes, P. J., Ito, K., Chung, K. F., Cavallesco, G., Azzena, G., Papi, A., Borea, P. A. (2006). Alteration of Adenosine Receptors in Patients with Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med*. 173:398–406.

Vaughan, K. R., Stokes, L., Prince, L. R., Marriott, H. M., Meis, S., Kassack, M. U., Bingle, C. D., Sabroe, I., Surprenant, A., Whyte, M. K. (2007). Inhibition of neutrophil apoptosis by ATP is mediated by the P2Y11 receptor. *J Immunol*. 179(12):8544-53.

Vestbo, J., Hurd, S. S., Agustí, A. G., Jones, P. W., Vogelmeier, C., Anzueto, A., Barnes, P. J., Fabbri, L. M., Martinez, F. J., Nishimura, M., Stockley, R. A., Sin, D. D., Rodriguez-Roisin, R. (2013). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med*. 187(4):347-65.

Vignola, A. M. (2004). PDE4 inhibitors in COPD—a more selective approach to treatment. *Respiratory medicine*. 98(6): 495–503.

Villunger, A., Scott, C., Bouillet, P. & Strasser, A. (2003). Essential role for the BH3-only protein Bim but redundant roles for Bax, Bcl-2, and Bcl-w in the control of granulocyte survival. *Blood*, 101, 2393-2400.

Vlahos, R., Wark, P. A. B., Anderson, G. P., Bozinovski, S. (2012). Glucocorticosteroids differentially regulate MMP-9 and neutrophil elastase in COPD. *PLoS ONE*. 7(3):e33277.

von Andrian, U. H., Chambers, J. D., McEvoy, L. M., Bargatze, R. F., Arfors, K. E., Butcher, E. C. (1991). Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins *In vivo*. *Proc Natl Acad Sci U S A.* 88(17):7538-42.

von Scheele, I., Larsson, K., Dahlén, B., Billing, B., Skedinger, M., Lantz, A.-S., Palmberg, L. (2011). Toll-like receptor expression in smokers with and without COPD. *Respiratory Medicine*. 105(8):1222–1230.

Wajant, H., Pfizenmaier, K., Scheurich, P. (2003). Tumor necrosis factor signaling. *Cell Death Differ*. 10(1):45–65.

Walchek, B., Kahn, J., Fisher, J. M., Wang, B. B., Fisk, R. S., Payan, D. G., Fehan, C., Betagari, R., Darlak, K., Spatola, A. F., Kishimoto, T. K. (1996). Neutrophil rolling altered by inhibition of L-selectin shedding *in vitro*. *Nature.* 380:720 –723.

Walker, B. A., Rocchini, C., Boone, R. H., Ip, S., Jacobson, M. A. (1997). Adenosine A2A receptor activation delays apoptosis in human neutrophils. *J Immunol*. 158(6):2926-31.

Wall, E. A., Zavzavadjian, J. R., Chang, M. S., Randhawa, B., Zhu, X., Hsueh, R. C., Liu, J., Driver, A., Bao, X. R., Sternweis, P. C., Simon, M. I., Fraser, I. D. (2009). Suppression of LPS-induced TNF-alpha production in macrophages by cAMP is mediated by PKA-AKAP95-p105. *Sci Signal*. 2(75):ra28.

Wallace, J. L., McKnight, W., Reuter, B. K., Vergnolle, N. (2000). NSAID-induced gastric damage in rats: requirement for inhibition of both cyclooxygenase 1 and 2. *Gastroenterology*. 119:706–714.

Wallace, A.E., Sales, K. J., Catalano, R. D., Anderson, R. A., Williams, A. R., Wilson, M. R., Schwarze, J., Wang, H., Rossi, A. G., Jabbour, H. N. (2009). Prostaglandin F2alpha-F-prostanoid receptor signaling promotes neutrophil chemotaxis via chemokine (C-X-C motif) ligand 1 in endometrial adenocarcinoma. *Cancer Res*. 69(14):5726-33.

Walmsley, S. R., Cowburn, A. S., Sobolewski, A., Murray, J., Farahi, N., Sabroe, I., Chilvers, E. R. (2004). Characterization of the survival effect of tumour necrosis factor-alpha in human neutrophils. *Biochem Soc Trans.* 32(3):456-60.

Walmsley, S. R., Print, C., Farahi, N., Peyssonnaux, C., Johnson, R. S., Cramer, T., Sobolewski, A., Condliffe, A. M., Cowburn, A. S., Johnson, N., Chilvers, E. R. (2005). Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. *J Exp Med*. 201(1):105-15.

Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., Xu, H., Walker, N. P., Perlmann, T. (2003). Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. *Nature*. 423(6939):555–560.

Wang, D., Cui, X. (2006). Evaluation of PDE4 inhibition for COPD. *Int J Chron Obstruct Pulmon Dis*. 1(4): 373–379.

Wang, G.G., Calvo, K.R., Pasillas, M.P., Sykes, D.B., Hacker, H. & Kamps, M.P. (2006). Quantitative production of macrophages or neutrophils ex vivo using conditional Hoxb8. *Nature Methods*, 3, 287-293.

Wang, S., Zhang, M., Liang, B., Xu, J., Xie, Z., Liu, C., Viollet, B., Yan, D., Zou, M. H. (2010). AMPKα2 deletion causes aberrant expression and activation of NAD(P)H oxidase and consequent endothelial dysfunction *In vivo*. Role of 26S proteasomes. *Circ Res.* 106:1117–1128.

Ward, C., Walker, A., Dransfield, I., Haslett, C., Rossi, A. G. (2004). Regulation of granulocyte apoptosis by NF-kappaB. *Biochem Soc Trans*. 32(Pt3):465-7.

Wardle, D. J., Burgon, J., Sabroe, I., Bingle, C. D., Whyte, M. K. B., Renshaw, S. A. (2011). Effective Caspase Inhibition Blocks Neutrophil Apoptosis and Reveals Mcl-1 as Both a Regulator and a Target of Neutrophil Caspase Activation. *PLoS One*. 6(1):e15768.

Wards, C., Dransfield, I., Murray, J., Farrow, S. Haslett, C., Rossi, A. G. (2002). Prostaglandin D2 and Its Metabolites Induce Caspase-Dependent Granulocyte Apoptosis That Is Mediated Via Inhibition of IκBα Degradation Using a Peroxisome Proliferator-

Activated Receptor-γ-Independent Mechanism. *The Journal of Immunology*. 168(12):6232-43.

Warren, H. S., Fitting, C., Hoff, E., Adib-Conquy, M., Beasley-Topliffe, L., Tesini, B., Liang, X., Valentine, C., Hellman, J., Hayden, D., Cavaillon, J. M. (2010). Resilience to bacterial infection: difference between species could be due to proteins in serum. *J. Infect. Dis*. 201(2):223–32.

Watson, R. W., Redmond, H. P., Wang, J. H., Condron, C., Bouchier-Hayes, D. (1996). Neutrophils undergo apoptosis following ingestion of Escherichia coli. *J. Immunol*. 156:3986–3992.

Watson, R. W. G., Rotstein, O. D., Nathens, A. B., Parodo, J., Marshall, J. C. (1997). Neutrophil apoptosis is modulated by endothelial transmigration and adhesion molecule engagement. *J. Immunol*. 158:945–953.

Watzer, B., Zehbe, R., Halstenberg, S., Kirkpatrick, J. C., Brochhausen, C. (2009). Stability of prostaglandin E(2) (PGE (2)) embedded in poly-D,L: -lactide-co-glycolide microspheres: a pre-conditioning approach for tissue engineering applications. *J Mater Sci Mater Med*. 20(6):1357-65.

Wedzicha, J. A., Seemungal, T. A., MacCallum, P. K., Paul, E. A., Donaldson, G. C., Bhowmik, A., Jeffries, D. J., Meade, T. W. (2000). Acute exacerbations of chronic obstructive pulmonary disease are accompanied by elevations of plasma fibrinogen and serum IL-6 levels. *Thromb Haemost*. 84:210–215.

Weih, F., Ryseck, R. P., Chen, L., Bravo, R. (1996). Apoptosis of nur77/N10-transgenic thymocytes involves the Fas/Fas ligand pathway. *Proc Natl Acad Sci USA*. 93:5533–5538.

Wheeldon, A., Vardey, C. J. (1993). Characterization of the inhibitory prostanoid receptors on human neutrophils. *Br J Pharmacol*. 108(4):1051-4.

Whyte, M. K. B., Meagher, L. C., MacDermot, J., Haslett, C. (1993a). Impairment of function in aging neutrophils is associated with apoptosis. *J. Immunol*. 150:5124–5134.

Whyte, M. K., Hardwick, S. J., Meagher, L. C., Savill, J. S., Haslett, C. (1993b). Transient elevations of cytosolic free calcium retard subsequent apoptosis in neutrophils *in vitro*. *J Clin Invest*. 92(1):446-55.

Willoughby, D. A., Adrian, R. M., Colville-Nash, P. R. (2000). COX-1, COX- 2 and COX-3 and the future treatment of chronic inflammatory disease. *Lancet*. 355:646–8.

Wilson, A. J., Arango, D., Mariadason, J. M., Heerdt, B. G., Augenlicht, L. H. (2003). TR3/Nur77 in colon cancer cell apoptosis. *Cancer Res*. 63(17):5401-7.

Witko-Sarsat, V., Halbwachs-Mecarelli, L., Schuster, A., Nusbaum, P., Ueki, I., Canteloup, S., Lenoir, G., Descamps-Latscha, B., Nadel, J. A. (1999). Proteinase 3, a potent secretagogue in airways, is present in cystic fibrosis sputum. *Am J Respir Cell Mol Biol*. 20: 729-736.

Wollin, L., Pieper, M. (2010) Tiotropium bromide exerts anti-inflammatory activity in a cigarette smoke mouse model of COPD. *Pulm Pharmacol Ther*. 23: 345–354.

Woo, M., Hakem, R., Soengas, M. S., Duncan, G. S., Shahinian, A., Kägi, D., Hakem, A., McCurrach, M., Khoo, W., Kaufman, S. A., Senaldi, G., Howard, T., Lowe, S. W., Mak, T. W. (1998). Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. *Genes Dev*. 12(6): 806–819.

Woronicz, J. D., Calnan, B., Ngo, V., Winoto, A. (1994). Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature*. 367: 277-281.

Xiang, B., Zhang, G., Guo, L., Li, X. A., Morris, A. J., Daugherty, A., Whiteheart, S. W., Smyth, S. S., Li, Z. (2013). Platelets protect from septic shock by inhibiting macrophage-dependent inflammation via the cyclooxygenase 1 signalling pathway. *Nat Commun*. 4:2657.

Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X. F., Achong, M. K. (1998). IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest*. 101(2):311-20.

Xu, M. H., Gong, Y. S., Su, M. S., Dai, Z. Y., Dai, S. S., Bao, S. Z., Li, N., Zheng, R. Y., He, J. C., Chen, J. F., Wang, X. T. (2011). Absence of the adenosine A2A receptor confers

pulmonary arterial hypertension and increased pulmonary vascular remodeling in mice. *J Vasc Res*. 48(2):171-83.

Xu, X. J., Reichner, J. S., Mastrofrancesco, B., Henry, W. L. Jr., Albina, J. E. (2008). Prostaglandin E2 suppresses LPS-stimulated IFNβ production. *J Immunol*. 180(4):2125–2131.

Yamagata, K., Matsumura, K., Inoue, W., Shiraki, T., Suzuki, K., Yasuda, S., Sugiura, H., Cao, C., Watanabe, Y., Kobayashi, S. (2001). Coexpression of microsomal-type prostaglandin E synthase with cyclooxygenase-2 in brain endothelial cells of rats during endotoxin-induced fever. *J Neurosci*. 21(8):2669-77.

Yamamoto, C., Yoneda, T., Yoshikawa, M., Fu, A., Tokuyama, T., Tsukaguchi, K., Narita, N. (1997). Airway inflammation in COPD assessed by sputum levels of interleukin-8. *Chest*. 112(2):505-10.

Yamane, H., Sugimoto, Y., Tanaka, S., Ichikawa, A. (2000). Prostaglandin E(2) receptors, EP2 and EP4, differentially modulate TNF-alpha and IL-6 production induced by lipopolysaccharide in mouse peritoneal neutrophils. *Biochem Biophys Res Commun*. 278(1):224-8.

Yang, D., de la Rosa, G., Tewary, P., Oppenheim, J. J. (2009). Alarmins link neutrophils and dentritic cells. *Trends in Immunology*. 30(11):531-537. Review.

Yang, J., Zheng, X., Haugen, F., Darè, E., Lövdahl, C., Schulte, G., Fredholm, B. B., Valen, G. (2014). Adenosine increases LPS-induced nuclear factor kappa B activation in smooth muscle cells via an intracellular mechanism and modulates it via actions on adenosine receptors. *Acta Physiol (Oxf)*. 210(3):590-9.

Yao, C., Hirata, T., Soontrapa, K., Ma, X., Takemori, H., Narumiya, S. (2013). Prostaglandin E_2 promotes Th1 differentiation via synergistic amplification of IL-12 signalling by cAMP and PI3-kinase. *Nat Commun*. 4:1685.

Yasui, K., Agematsu, K., Shinozaki, K., Hokibara, S., Nagumo, H., Nakazawa, T., Komiyama, A. (2000). Theophylline induces neutrophil apoptosis through adenosine A2A receptor antagonism. *J Leukoc Biol*. 67(4):529-35.

Yokose, N., Ogata, K., Tamura, H., An, E., Nakamura, K., Kamikubo, K., Kudoh, S., Dan, K., Nomura, T. (1998). Pulmonary Toxicity after granulocyte colony-stimulating factor-combines chemotherapy for non-Hodkin's lymphoma. *British Journal of cancer.* 77(12):2286-2290.

Yoshida, T., Tuder, R. M. (2007). Pathobiology of cigarette smoke-induced chronic obstructive pulmonary disease. *Physiol Rev*. 87(3):1047-82.

Yoshioka, A., Betsuyaku, T., Nishimura, M., Miyamoto, K., Kondo, T., Kawakami, Y. (1995). Excessive neutrophil elastase in bronchoalveolar lavage fluid in subclinical emphysema. *Am J Respir Crit Care Med*. 152(6 Pt 1):2127-32.

Yusta, B., Ortiz-Caro, J., Pascual, A., Aranda, A. (1988) Comparison of the effects of forksolin and dibutyryl cyclic AMP in neuroblastoma cells evidence that some of the actions of dibutyryl cyclic AMP are mediated by butyrate. *Journal of Neurochemistry*. 51:1808-1818.

Zaccolo, M., Pozzan, T. (2002) Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science*. 295:1711-1715.

Zeid, N. A., Muller, H. K. (1995). Tobacco smoke induced lung granulomas and tumors: association with pulmonary Langerhans cells. *Pathology*. 27:247-254.

Zhang, Y., Broser, M., Cohen, H., Bodkin, M., Law, K., Reibman, J., Rom, W. N. (1995). Enhanced interleukin-8 release and gene expression in macrophages after exposure to Mycobacterium tuberculosis and its components. *J Clin Invest*. 95(2):586–592.

Zhang, J., DeYoung, A., Kasler, H. G., Kabra, N. H., Kuang, A. A., Diehl, G., Sohn, S. J., Bishop, C., Winoto, A. (1999). Receptor-mediated apoptosis in T lymphocytes. *Cold Spring Harb Symp Quant Biol*. 64:363–371.

Zhang, X., Moilanen, E., Kankaanranta, H. (2001). Beclomethasone, budesonide and fluticasone propionate inhibit human neutrophil apoptosis. *Eur J Pharmacol*. 431:365-371.

Zhang, X., Moilanen, E., Adcock, I. M., Lindsay, M. A., Kankaanranta, H. (2002). Divergent effect of mometasone on human eosinophil and neutrophil apoptosis. *Life Sci*. 71:1523–1534.

Zhang, B., Hirahashi, J., Cullere, X., Mayadas, T. N. (2003). Elucidation of molecular events leading to neutrophil apoptosis following phagocytosis: cross-talk between caspase 8, reactive oxygen species, and MAPK/ERK activation. *J. Biol. Chem.* 278:28443–28454.

Zhang, L., Paine, C., Dip, R. (2010). Selective regulation of nuclear orphan receptors 4A by adenosine receptor subtypes in human mast cells. *J Cell Commun Signal*. 4(4):173-83.

Zhang, J., Hel, J., Xia, J., Chen, Z., Chen, X. (2012). Delayed apoptosis by neutrophils from COPD patients is associated with altered bak, bcl-xl, and mcl-1 mRNA expression. *Diagnostic Pathology*. 7:65.

Zhao, X., Zmijewski, J. W., Lorne, E., Liu, G., Park, Y.-J., Tsuruta, Y., Abraham, E. (2008). Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. *Am J Physiol Lung Cell Mol Physiol.* 295(3):L497–L504.

Zhao, Y., Bruemmer, D. (2010). NR4A Orphan Nuclear Receptors: Transcriptional Regulators of Gene Expression in Metabolism and Vascular Biology. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 30: 1535-1541.

Zhao, Y., Cui, A., Wang, F., Wang, X. J., Chen, X., Jin, M. L., Huang, K. W. (2012). Characteristics of pulmonary inflammation in combined pulmonary fibrosis and emphysema. *Chin Med J (Engl)*. 125(17):3015-21.

Zhu, J. I. E., Qiu, Y. S., Majumdar, S., Gamble, E., Matin, D., Turato, G., Fabbri, L. M., Barnes, N., Saetta, M., Jeffery, P. K. (2001). Exacerbations of bronchitis, bronchial eosinophilia and gene expression for interleukin-4, interleukin-5, and eosinophil chemoattractants. *Am J Respir Crit Care Med*. 164:109–116.

Zinovkin, R. A., Romaschenko, V. P., Galkin, I. I., Zakharova, V. V., Pletjushkina, O. Y., Chernyak, B. V., Popova, E. N. (2014). Role of mitochondrial reactive oxygen species in age-related inflammatory activation of endothelium. *Aging (Albany NY)*. 6(8):661-74.

Zlotnik, A. & Yoshie, O. (2000) Chemokines: A new classification system and their role in immunity. *Immunity*. 12:121-127.

CHAPTER 8. APPENDICES

Appendix 1. Materials

Table 8: Table of reagents used in this study.

Description	Reagent	Supplier	Location
Receptor Agonists/Antagonists			
EP1/2/3/4 agonist	PGE2	Cayman Chemical	Ann Arbor, MI
EP1/2/3/4 agonist	16,16-dimethyl PGE2	Cayman Chemical	Ann Arbor, MI
EP1/3 agonist	17 phenyl trinor PGE2	Cayman Chemical	Ann Arbor, MI
EP2 agonist	®-Butaprost (free acid)	Sigma	St. Louis, MO
EP2 antagonist	PF-04418948	Cayman Chemical	Ann Arbor, MI
EP3 agonist	Sulprostone	Cayman Chemical	Ann Arbor, MI
EP4 agonist	L-902,688	Cayman Chemical	Ann Arbor, MI
EP4 antagonist	GW 627368X	Cayman Chemical	Ann Arbor, MI
FP agonist	PGF2α	Cayman Chemical	Ann Arbor, MI
FP antagonist	AL-8810	Cayman Chemical	Ann Arbor, MI
PPARγ agonist	Rosiglitazone	Cayman Chemical	Ann Arbor, MI
PPARγ antagonist	GW9662	Cayman Chemical	Ann Arbor, MI
A1/A2/A3 agonist	Adenosine	Sigma	St. Louis, MO
A2A antagonist	ZM 241385	Sigma	St. Louis, MO
TLR4 agonist	LPS	Enzo Life Sciences	Exeter, UK
Pharmacological Modulators of Neutrophil Apoptosis			
cAMP analogue	dbcAMP	Sigma	St. Louis, MO
Site-selective PKA agonist (Site A of RIα)	N6-MB-cAMP	Sigma	St. Louis, MO
Site-selective PKA agonist (Site B of RIα)	8-AHA-cAMP	Sigma or Calbiochem/CN Biosciences	St. Louis, MO or Beeston, UK
PKA antagonist (Site A & B of RIα, Site A of RIIα)	Rp-8-Br-cAMPS	Biolog	Hayward, CA
Protein Kinase Inhibitor	Staurosporine	Sigma	St. Louis, MO
Selective PDE4 inhibitor	Rolipram	Cayman Chemical	Ann Arbor, MI
Adenylyl cyclase inhibitor	SQ 22,536	Cayman Chemical	Ann Arbor, MI
Cytokines			

Recombinant cytokine	Murine GM-CSF	Peprotech	Rocky Hill, NJ
Recombinant cytokine	Murine SCF	Peprotech	Rocky Hill, NJ
Cell Culture			
Cell medium	RPMI Medium 1640	Gibco	Waltham, MA
Cell medium	OptiMEM	Invitrogen	Paisley, UK
Serum	Heat Inactivated FCS (Catalogue #C-37372; Lot #07372381)	Promocell	Heidelberg, Germany
Estrogen	β-estradiol	Sigma	St. Louis, MO
Flexiwell plates	96 well Flexiwell plates	Costar (corning inc.)	Amsterdam, The Netherlands
Amino Acid	L-Glutamine	Invitrogen	Paisley, UK
Cell Dissociation	Non-enzymatic cell dissociation solution	Sigma	St. Louis, MO
Antioxidant	β-mercaptoethanol	Sigma	St. Louis, MO
Antibiotics	Penicillin/Streptomycin	Invitrogen/Life Technologies	Paisley, UK
Culture flask	T25 cm²/ T75 cm² flasks	Nunc(lon)	Roskilde, Denmark
Solvent	DMSO	Sigma	St. Louis, MO
Neutrophil isolation			
Anticoagulant	Sodium citrate BP	Martindale	Brentwood, UK
Sedimentation	Dextran	Sigma	St. Louis, MO
Density Separation	Percoll	Sigma	St. Louis, MO
Density Separation	Histopaque	Sigma	St. Louis, MO
Buffer	1x HBSS without Ca^{2+}/Mg^{2+}	Gibco	Waltham, MA
Base	7.5% sodium bicarbonate	Gibco	Waltham, MA
Antibody cocktail	Human StemSep Negative Human Granulocyte Cocktail Lot 12H45799	StemCell Technologies	Vancouver, Canada
Cell separation reagent	StemSep magnetic colloid	StemCell Technologies	Vancouver, Canada
Cell separation column	LS Columns	Miltenyi Biotech	Bisley, UK
Apoptosis Assay			
Cell stain	Diff-Quik stains	Reagena	Toivala, Finland
Centrifuge	Cytospin centrifuge	Shandon Inc.	Pittsburgh, PA

Mounting Medium	DPX	Fisher Chemical	Loughborough, UK
Microscope	Light Microscope	Nikon	Surrey, UK
Flow Cytometry Reagents			
Buffer	Annexin Binding Buffer	BD Pharmigen	San Diego, CA
Calcium chelator	0.5M EDTA, pH 8.0	Invitrogen/Life Technologies	Paisley, UK
Fluorescent beads	CountBright Absolute Beads	Invitrogen	Paisley, UK
Flow cytometer	FACSCalibur	Becton Dickinson	San Jose, CA
PS stain	PE-annexin V	BD Pharmigen	San Diego, CA
Viability stain	ToPro-3	Molecular Probes	Paisley, UK
Software	CellQuest Pro	BD Biosciences	San Jose, CA
Software	FlowJo	FlowJo, LLC	Ashland, OR
Transfection Reagents			
Electroporator	Amaxa Nucleofector Kit V	Lonza	Basel, Switzerland
siRNA pool	ON-TARGET plus cyclophilin B control siRNA	Dharmacon (Thermo Fisher Scientific)	Lafayette, CO
siRNA pool	ON-TARGET plus non-targeting pool siRNA	Dharmacon (Thermo Fisher Scientific)	Lafayette, CO
Mcl-1 siRNA pool	L-062229-00-0005	Dharmacon (Thermo Fisher Scientific)	Lafayette, CO
Casp3 siRNA pool	L-043042-00-0005	Dharmacon (Thermo Fisher Scientific)	Lafayette, CO
NR4A2 siRNA pool	L-048281-01-0005	Dharmacon (Thermo Fisher Scientific)	Lafayette, CO
NR4A3 siRNA pool	L-043983-01-0005	Dharmacon (Thermo Fisher Scientific)	Lafayette, CO
RNA Extraction			
RNA isolation reagent	TRI reagent LS	Sigma	St. Louis, MO
Phase separation reagent	1-bromo-3 chloropropane	Santa Cruz Chemical	Dallas, TX
DNAse treatment kit	DNA-free DNAse Treatment & Removal Kit	Ambion (Life Technologies)	Carlsbad, CA
RT kit	High-Capacity cDNA Reverse Transcription Kit	Applied Biosciences	Carlsbad, CA
Alcohol	Isopropanol	(BDH) VWR International or Fisher-Chemical	Poole, UK or Loughborough, UK
Alcohol	Methanol	(BDH) VWR International	Poole, UK
qPCR Reagents			

qPCR Buffer Mix	qPCR MasterMix Plus	Eurogentec	Seraing, Belgium
GAPDH plasmid	IRAU24E08	Source BioScience	Rochdale, UK
EP4 plasmid	IRATp970E11120D	Source BioScience	Nottingham, UK
GAPDH Primer/Probe Set	Hs02758991_g1	Applied Biosystems	Foster City, CA
NR4A2 Primer/Probe Set	Hs00428691_m1	Applied Biosystems	Foster City, CA
NR4A3 Primer/Probe Set	Hs00545009_g1	Applied Biosystems	Foster City, CA
PTGER2 (EP2) Primer/Probe Set	Hs04183523_m1	Applied Biosystems	Foster City, CA
PTGER4 (EP4) Primer/Probe Set	Hs00168761_m1	Applied Biosystems	Foster City, CA
RT-PCR Reagents			
PCR reagent	25 mM MgCl2	Promega	Southampton, UK
dNTP	dATPs, dTTPs, dGTPs, dCTPs,	Promega	Southampton, UK
Polymerase	GoTaq DNA polymerase	Promega	Southampton, UK
Buffer	Green GoTaq Flexi Buffer	Promega	Southampton, UK
Marker	HyperLadder I	Bioline	London, UK
Marker	HyperLadder IV	Bioline	London, UK
Software	ImageJ	Public Domain	N.A.
PCR primer	PCR Primer	Sigma	St. Louis, MO
Marker	PCR Ranger 100bp DNA Ladder	Norgen Biotek Corporation	Thorold, Canada
Polysaccharide polymer	Agarose	Melford	Suffolk, UK
DNA intercalating agent	Ethidium Bromide	Sigma	St. Louis, MO
Chemiluminescence imager	Chemidoc XRS+	BioRad	Hercules, CA
Western Blotting Reagents			
Serine protease inhibitor	DFP	Sigma	St. Louis, MO
Serine protease inhibitor	PMSF	Sigma	St. Louis, MO
Protease inhibitor	Protease Inhibitor	Calbiochem	St. Louis, MO
Blocking agent	BSA 100mg/ml	Promega	Southampton, UK

Blocking agent	Milk	Sainsbury's	London, UK
Blotting membrane	Nitrocellulose	Biotrace	Onehunga, New Zealand
Marker	Colour Protein Standard Broad Range	New England BioLabs	Ipswich, MA
Primary antibody	EP2 antibody (H-75)	Santa Cruz Biotechnology	Dallas, TX
Primary antibody	EP4 antibody (C-4)	Santa Cruz Biotechnology	Dallas, TX
Primary antibody	Actin primary (mouse)	Dako	Glostrup, Denmark
Secondary antibody	Polyclonal Anti-rabbit IgG-HRP	Dako	Glostrup, Denmark
Secondary antibody	Polyclonal Anti-mouse IgG-HRP	Dako	Glostrup, Denmark
Protein stain	Ponceau S	Sigma	St. Louis, MO
Peroxidase substrate	Clarity Western ECL substrate	BioRad	Hercules, CA
Various Reagents			
Buffer	10x DPBS	Invitrogen	Paisley, UK

Pharmaceuticals were dissolved in PBS or dimethyl sulfoxide (DMSO) and further diluted in PBS to obtain a final concentration of DMSO < 0.1 %.

Appendix 2. Buffer Recipes

- **Gel Electrophoresis**

Recipe for 50x TAE Buffer

Reagent	Concentration
TRIS base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100 ml

Recipe for 1x TAE Buffer

20 ml 50x TAE Buffer + 980 ml ddH_20

Recipe for 1 % Agarose Gel

Reagent	Amount
Agarose powder	1.5 g
1x TAE	100 ml
Ethidium Bromide (µl)	0.8 µl

- **Western Blotting Buffers**

Recipe for PMN lysis buffer

Reagent	Concentration
Diisopropylfluorophosphate (DFP)	1 mM (1:100 of 100 mM stock)
Phenylmethanesulfonylfluoride (PMSF)	1 mM (1:100 of 100 mM stock)
PI (protease inhibitor set)	1 mM (1:100 of 100 mM stock)
H_20	485 µl

Recipe for 2x SDS loading dye

Reagent	Concentration
TRIS-Cl (pH 6.8)	100 mM
SDS	4 % (w/v)
Bromophenol Blue	0.2 % (w/v)
Glycerol	20 % (w/v)

β-mercaptoethanol	200 mM

Recipe for 10x Running Buffer

Reagent	Concentration
TRIS base	30.3 g
Glycine	190 g
20 % SDS	50 ml
ddH_20	to 1 L

Recipe for 1x Running Buffer

100 ml 10x Running Buffer + 900 ml ddH_20

Recipe for 1x Transfer Buffer

Reagent	Concentration
TRIS base	5.8 g
Glycine	2.9 g
20 % SDS	1.85 ml
MeOH	200 ml
ddH_20	to 1 L

Recipe for 1x Blocking Buffer

Reagent	Concentration
Skimmed milk powder	1.5 g
1x TBS	30 ml

Recipe for 10x TBS-Tween

Reagent	Concentration
Tris HCl (1M, pH 8.0)	100 ml
NaCl (1M)	97.3 g
Tween 20	2.5 ml
ddH_20	to 1 L

Recipe for 1x TBS-Tween

100 ml 10x TBS-Tween + 900 ml ddH_20

Recipe for 10% Resolving Gel (SDS-PAGE)

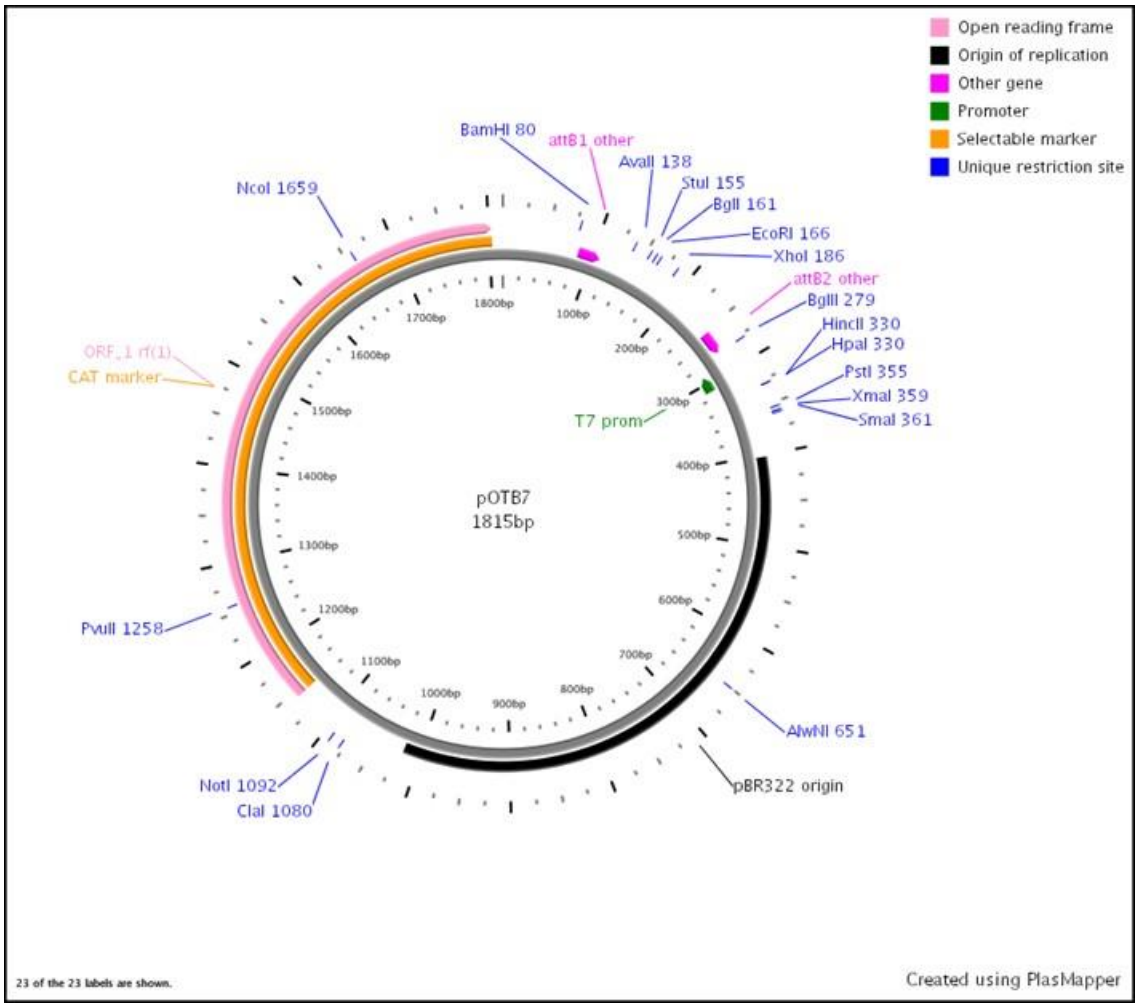
Reagent	Volume
ddH_20	3.2 ml
30 % Acrylamide	2.67 ml
1.5 M Tris (pH 8.8)	2 ml
10 % SDS	80 µl
10 % Ammonium Persulphate (APS)	80 µl
TEMED	8 µl
Total Volume	8 ml

Recipe for 6 % Stacking Gel (SDS-PAGE)

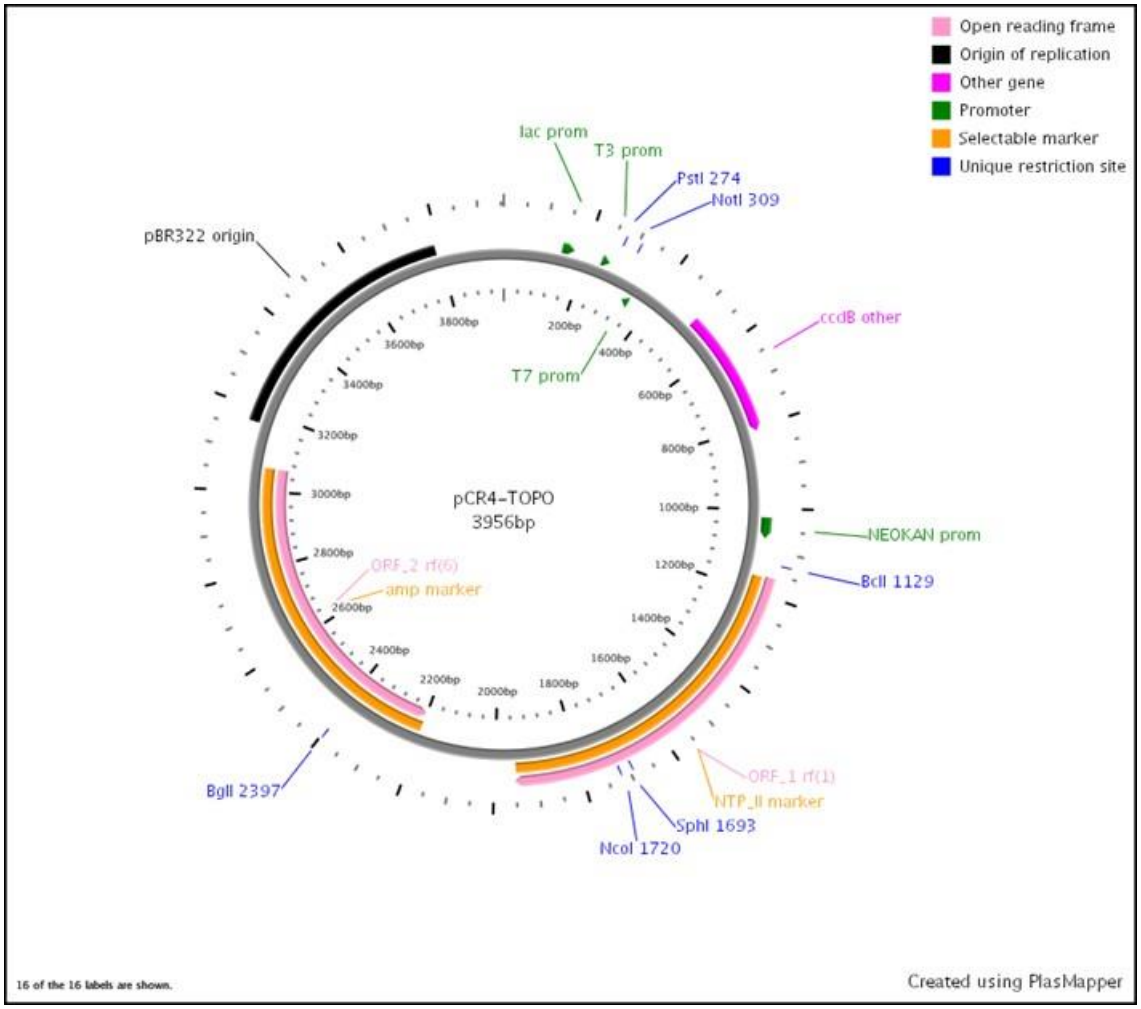
Reagent	Volume
ddH_20	2.6 ml
30 % Acrylamide	1 ml
0.5 M Tris (pH 6.8)	1.25 ml
10 % SDS	50 µl
10 % Ammonium Persulphate (APS)	50 µl
TEMED	5 µl
Total Volume	5 ml

Appendix 3. Vector Map

- GAPDH Base vector pOTB7 (Source Bioscience)/IRAU24E08



- EP2/EP4 Base vector MAM pCR4-TOPO (Source Bioscience)/ IRATp970E11120D



Appendix 3. DNA Sequences

- ***GAPDH* sequence and location of primer targeting sequences (yellow).**

GGGGTGGGCCCGGGCGGCCTCCGCATTGCAGGGGCGGGCGGAGGACGTGA
TGCGGCGCGGGCTGGGCATGGAGGCCTGGTGGGGGAGGGGAGGGGAGGCG
TGTGTGTCGGCCGGGGCCACTAGGCGCTCACTGTTCTCTCCCTCCGCGCAGC
CGAGCCACATCGCTCAGACACCATGGGGAAGGTGAAGGTCGGAGTCAACG
GATTTGGTCGTATTGGGCGCCTGGTCACCAGGGCTGCTTTTAACTCTGGTAA
AGTGGATATTGTTGCCATCAATGACCCCTTCATTGACCTCAACTACATGGTT
TACATGTTCCAATATGATTCCACCCATGGCAAATTCCATGGCACCGTCAAGG
CTGAGAACGGGAAGCTTGTCATCAATGGAAATCCCATCACCATCTTCCAGG
AGCGAGATCCCTCCAAAATCAAGTGGGGCGATGCTGGCGCTGAGTACGTCG
TGGAGTCCACTGGCGTCTTCACCACCATGGAGAAGGCTGGGGCTCATTTGC
AGGGGGGAGCCAAAAGGGTCATCATCTCTGCCCCCTCTGCTGATGCCCCCA
TGTTCGTCATGGGTGTGAACCATGAGAAGTATGACAACAGCCTCAAGATCA
TCAGCAATGCCTCCTGCACCACCAACTGCTTAGCACCCCTGGCCAAGGTCAT
CCATGACAACTTTGGTATCGTGGAAGGACTCATGACCACAGTCCATGCCAT
CACTGCCACCCAGAAGACTGTGGATGGCCCCTCCGGGAAACTGTGGCGTGA
TGGCCGCGGGGCTCTCCAGAACATCATCCCTGCCTCTACTGGCGCTGCCAAG
GCTGTGGGCAAGGTCATCCCTGAGCTGAACGGGAAGCTCACTGGCATGGCC
TTCCGTGTCCCCACTGCCAACGTGTCAGTGGTGGACCTGACCTGCCGTCTAG
AAAAACCTGCCAAATATGATGACATCAAGAAGGTGGTGAAGCAGGCGTCG
GAGGGCCCCCTCAAGGGCATCCTGGGCTACACTGAGCACCAGGTGGTCTCC
TCTGACTTCAACAGCGACACCCACTCCTCCACCTTTGACGCTGGGGCTGGCA
TTGCCCTCAACGACCACTTTGTCAAGCTCATTTCCTGGTATGACAACGAATT
TGGCTACAGCAACAGGGTGGTGGACCTCATGGCCCACATGGCCTCCAAGGA
GTAAGACCCCTGGACCACCAGCCCCAGCAAGAGCACAAGAGGAAGAGAGA
GACCCTCACTGCTGGGGAGTCCCTGCCACACTCAGTCCCCCACCACACTGA
ATCTCCCCTCCTCACAGTTGCCATGTAGACCCCTTGAAGAGGGGAGGGGCC
TAGGGAGCCGCACCTTGTCATGTACCATCAATAAAGTACCCTGTGCTCAACC
AGTTAAAAAAAAAAAAAAAAAAAA

- **EP2 protein sequence and location of targeting sequence (yellow).**

MGNASNDSQSEDCETRQWLPPGESPAISSVMFSAGVLGNLIALALLARRWRGD
VGCSAGRRSSLSLFHVLVTELFTDLLGTCLISPVVLASYARNQTLVALAPESRAC
TYFAFAMTFFSLATMLMLFAMALERYLSIGHPYFYQRRVSRSGGLAVLPVIYA
VSLLFCSLPLLDYGQYVQYCPGTWCFIRHGRTAYLQLYATLLLLLIVSVLACNF
SVILNLIRMHRRSRRSRCGPSLGSGRGGPGARRRGERVSMAEETDHLILLAIMTI
TFAVCSLPFTIFAYMNETSSRKEKWDLQALRFLSINSIIDPWVFAILRPPVLRLMR
SVLCCRISLRTQDATQTSCSTQSDASKQADL

- **EP4 protein sequence and location of targeting sequence (yellow).**

MSTPGVNSSASLSPDRLNSPVTIPAVMFIFGVVGNLVAIVVLCKSRKEQKETTFY
TLVCGLAVTDLLGTLLVSPVTIATYMKGQWPGGQPLCEYSTFILLFFSLSGLSIIC
AMSVERYLAINHAYFYSHYVDKRLAGLTLFAVYASNVLFCALPNMGLGSSRL
QYPDTWCFIDWTTNVTAHAAYSYMYAGFSSFLILATVLCNVLVCGALLRMHR
QFMRRTSLGTEQHHAAAAASVASRGHPAASPALPRLSDFRRRRSFRRIAGAEIQ

MVILLIATSLVVLICSIPLVVRVFVNQLYQPSLEREVSKNPDLQAIRIASVNPILDP
WIYILLRKTVLSKAIEKIKCLFCRIGGSRRERSGQHCSDSQRTSSAMSGHSRSFIS
RELKEISSTSQTLLPDLSLPDLSENGLGGRNLLPGVPGMGLAQEDTTSLRTLRISE
TSDSSQGQDSESVLLVDEAGGSGRAGPAPKGSSLQVTFPSETLNLSEKCI

Appendix 4. Primer Sequences

Table 9: Nucleotide sequences and specifications of conventional PCR primers by Sigma.

Primer	Sequence (5' -> 3')	Length	Tm (°C)	GC%	Product	Anneal (°C)
Murine *GAPDH* (Fwd)	GGTGAAGGTCG GTGTGAACG	20	61.22	60	233 (transcript variants 1, 2, X1, X2, X3)	56
Murine *GAPDH* (Rev)	CTCGCTCCTGG AAGATGGTG	20	60.18	60		
Murine *CypB* (Fwd)	TCGGAGCGCAA TATGAAGGTGC	22	63.54	54.55	343	58
Murine *CypB* (Rev)	AGATGCTCTTTC CTCCTGTGCCA	23	63.81	52.17		
Murine *Casp3* (Fwd)	CTCGCTCTGGTA CGGATGTG	20	65.8	60	379 (transcript variants 1, 2)	61
Murine *Casp3* (Rev)	ACCGGTATCTTC TGGCAAGC	20	65.3	55		
Murine *Mcl-1* (Fwd)	TGTAAGGACGA AACGGGACT	20	63.4	50	265	59
Murine *Mcl-1* (Rev)	AAATCCTGGGC AGCTTCAAGT	21	65.9	47.6		
Murine *NR4A2* (Fwd)	GACGGGCTGGA TTCCCAATA	20	68	55	990 (transcript variants 1, 2, X1, X2)	62
Murine *NR4A2* (Rev)	GCACCGTGCGC TTAAAGAAA	20	67.2	50		
Murine *NR4A3* (Fwd)	TTCTGACGGCCT CCATTGAC	20	67.5	55	328	62
Murine *NR4A3* (Rev)	AACCCATGTCG CTCTGTGAT	20	64.6	50		
Human *GAPDH* (Fwd)	GGTGAAGGTCG GAGTCAACG	20	61.22	60	233 (transcript variants 1, 3, 4)	56
Human *GAPDH* (Rev)	CTCGCTCCTGG AAGATGGTG	20	60.18	60		
Human *PTGER1* (Fwd)	ATCATGGTGGT GTCGTGCAT	20	60.04	50	149	56
Human *PTGER1* (Rev)	TACACCCAAGG GTCCAGGAT	20	59.88	55		
Human *PTGER2* (Fwd)	CAACCTCATCCG CATGCAC	19	59.57	57.89	419	55
Human *PTGER2* (Rev)	CTCAAAGGTCA GCCTG	16	50.92	56.25		
Human *PTGER3* (Fwd)	CGCCTCAACCA CTCCTACACA	21	62.04	57.14	837 (transcript variants 4, 5, 6, 7, 8, 9, 11)	61
Human *PTGER3* (Rev)	GCAGACCGACA GCACGCACAT	21	66.07	61.9		
Human *PTGER4* (Fwd)	TGGTATGTGGG CTGGCTG	18	59.32	61.11	434 (transcript variants 1, X1, X2)	55
Human *PTGER4* (Rev)	GAGGACGGTGG CGAGAAT	18	59.11	61.11		

Appendix 5. siRNA Sequences

Table 10: Nucleotide sequences of ON-TARGETplus SMARTpool siRNA by Thermo Scientific.

Primer		Sequence (5' -> 3')
Mcl1	J-062229-05	UUUCAAAGAUGGCGUAACA
	J-062229-06	AAACGAAGGCGAUGUUAAA
	J-062229-07	CCGAAAGGCGGCUGCAUAA
	J-062229-08	GAUCAUCUCGCGCUACUUG
CypB	Mix of 4 proprietary siRNAs	
Casp3	J-043042-05	GAAAUGGGCAUAUGCAUAA
	J-043042-06	CAACGGAAUUCGAGUCCUU
	J-043042-07	GGAUAGUGUUUCUAAGGAA
	J-043042-08	CGCACAAGCUAGAAUUUAU
NR4A2	J-048281-09	CGUCAGAGCCCACGUCGAU
	J-048281-10	CCACACAGCGGGUCGGUUU
	J-048281-11	AGGCAAACCCUGACUAUCA
	J-048281-12	CAGAAUAUGAACAUCGACA
NR4A3	J-043983-09	AGACUUAUGGCUCGGAAUA
	J-043983-10	CAAGAGACGCCGAAACCGA
	J-043983-11	GGUUAAGGAAGUUGUGCGU
	J-043983-12	GAUGAAUGCCCUUGUCCGA

Appendix 6. Meetings

- Powerpoint Presentation at the **Infection and Immunity Research Day, Sheffield (2015).**

  Lipopolysaccharide Upregulates Prostaglandin Receptor EP2 mRNA Expression in Human Peripheral Blood Neutrophils from COPD Patients and Healthy Controls. Authors: Svenja Dannewitz, Lynne Prince, Ian Sabroe, David Sammut, Richard Budd, David Dockrell, Moira Whyte.

- Powerpoint Presentation at the **Yorkshire Immunology Group Meeting, Leeds (2014).**

  Prostaglandin E2 Regulates NR4A2 and NR4A3 through cAMP/PKA Signalling in Human Peripheral Blood Neutrophils. Authors: Svenja Dannewitz, Elizabeth Jones, Lynne Prince, Ian Sabroe, Moira Whyte.

- Poster Presentation at the **University of Sheffield Medical School 10th Annual Research Meeting, Sheffield (2014).**

  The role of orphan nuclear receptors NR4A2 and NR4A3 in neutrophil cell death. Authors: Svenja Dannewitz, Lynne Prince, Ian Sabroe, Moira Whyte.

- Poster Presentation at the **Department of Infection and Immunity Annual Research Day, Sheffield (2013).**

  Targeting PKA pathways: A potential strategy in inflammatory disease. Authors: Svenja Dannewitz, Lynne Prince, Ian Sabroe, Moira Whyte.

- Poster Presentation at the **University of Sheffield Medical School 8th Annual Research Meeting, Sheffield (2012).**

  Murine Haematopoietic Stem Cells: A Model to Investigate Pathways Underpinning Neutrophil Apoptosis and Survival. Authors: Svenja Dannewitz, Lynne Prince, Ian Sabroe, Moira Whyte.